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Abstract

This study investigated the effects of dietary supplementation with a coated complex of essential oils, organic acids, vitamin E, and selenium on growth performance, intestinal health, immune responses, and gut microbiota in Escherichia coli-challenged weaned pigs. Twenty-four crossbred (Landrace × Yorkshire × Duroc) weaned pigs (12 barrows and 12 gilts; 4 weeks of age; initial body weight 9.61 ± 0.81 kg) were randomly assigned to four treatments in a completely randomized design, with six replicates per treatment and one pig per pen, for 14 days experimental period. The dietary treatments were as follows: non-challenged control (PC), challenged control (NC), NC with 0.05% additive (T1), and NC with 0.10% additive (T2). Pigs in NC, T1, and T2 were orally challenged with *E. coli* K88 on days -2 to 0. On day 7, body weight was significantly higher in T1 and T2 than NC ($p < 0.05$), and average daily gain was greater in T1 than NC during days 0–7 ($p < 0.05$). Fecal scores were significantly lower in PC, T1, and T2 than NC during weeks 1 and 2 ($p < 0.05$). Apparent total tract digestibility of dry matter, crude protein, and gross energy did not differ among treatments ($p > 0.05$). Ileal villus height to crypt depth ratio was significantly higher in PC, T1, and T2 than NC ($p < 0.05$). Expression of claudin-1 and mucin-1 was significantly higher in T1 and T2 than NC and PC ($p < 0.05$), while zonula occludens-1 expression was higher in T1 than NC ($p < 0.05$). On day 3 post-challenge, serum interferon-γ was significantly higher in T1 and T2 than PC ($p < 0.05$), and interleukin-8 was elevated in T2 compared to PC ($p < 0.05$). Beta diversity analysis revealed significant differences in microbial community structure between treatments. At the genus level, T2 showed significantly higher relative abundances of Lactobacillus and Megasphaera than other groups ($p < 0.05$). In conclusion, dietary supplementation with this coated complex at 0.05% and 0.10% improved growth performance, intestinal morphology, barrier function, and immune responses in *E. coli*-challenged weaned pigs.

Keywords (3 to 6): essential oils, organic acids, Vitamin E, selenium, weaned pigs, *Escherichia coli* challenge

INTRODUCTION

Weaning is a common practice used to enhance economic efficiency in pigs; however, it exposes them to various stress factors [1]. Weaning stress leads to oxidative stress, which damages intestinal development [1]. These effects enhance inflammatory responses to pathogens, leading to post-weaning diarrhea (PWD), which reduces growth performance and increases mortality [2]. Nutritional strategies aimed at reducing stress related to PWD, while also supporting intestinal health and immune function, are essential during this period.

Antibiotics have traditionally been used to tackle weaning challenges in pigs [3]. Recently, increasing restrictions on antibiotic use have limited their application, leading to growing interest in functional additives as potential alternatives [3]. Among the functional compounds, essential oils (EO) are bioactive substances known for their antioxidant and anti-inflammatory properties. They help modulate the intestinal environment and immune responses during weaning stress [4]. Organic acid (OA) supports digestive health and enhances the immune response in immunocompromised weaned pigs by reducing pH and modulating gut microbiota [5]. Additionally, vitamin E and selenium act as antioxidant additives that help alleviate oxidative stress associated with weaning. Previous studies have shown that vitamin E supplementation reduces serum immune cell function and enhances total antioxidant capacity, while selenium improves antioxidant status and boosts glutathione peroxidase activity in weaned pigs facing oxidative challenges [6,7]. Functional additives have been extensively studied for individual supplementation; however, their varying functional mechanisms indicate potential synergistic effects when used in combination [8]. A previous study reported that dietary supplementation with a combination of EO and OA improved final body weight (BW), increased average daily gain (ADG), and enhanced fecal isovaleric acid levels in weaned pigs [9]. Additionally, Liu et al. [10] reported that dietary supplementation with vitamin E and selenium increased glutathione peroxidase activity, reduced the ratio of oxidized to reduced glutathione, and mitigated the effects of oxidative stress on intestinal barrier integrity.

However, gastric degradation and early absorption limit the delivery of these compounds to the lower intestinal tract [11]. Encapsulation is a technique that involves coating small solid particles, liquid droplets, or gases with a protective layer. This method has been developed to protect compounds from gastric degradation and to ensure the targeted delivery of substances to specific sites within the gastrointestinal tract [12]. Dietary supplementation with microencapsulated OA and EO at 1 g/kg improved the gain-to-feed ratio (G:F) compared to the free acidifier treatment, while 2 g/kg supplementation increased ADG in *E. coli*-challenged pigs [13]. In a separate study, an encapsulated mixture of OA and EO at 1,000 to 2,000 mg/kg increased ADG and reduced diarrhea frequency

compared to a control diet, indicating effects that were comparable to or better than those of antibiotic growth promoters in weaned pigs [14].

Despite the reported benefits of encapsulation technology and the potential synergies among bioactive compounds, no studies have assessed the combined effects of EO, OA, vitamin E, and selenium in an encapsulated formulation. This study, therefore, aimed to evaluate the impact of an encapsulated additive complex on growth performance, nutrient digestibility, intestinal health, immune responses, and intestinal microbiota in weaned pigs challenged with *Escherichia coli* (*E. coli)*.

MATERIALS AND METHODS

Ethics approval and consent to participate

The protocol for this study was approved by the Institutional Animal Care and Use Committee of Chungbuk National University, Cheongju, Republic of Korea (approval no. CBNUA-24-0009-01).

Bacterial strains, culture and challenge

E. coli K88 (KCTC 2571) was obtained from the Korean Collection for Type Cultures (KCTC, Jeongeup, Korea) in lyophilized form and reconstituted in sterile distilled water. An aliquot of 10 μL of the reconstituted culture was inoculated into Luria-Bertani broth (LB broth; KisanBio, Seoul, Korea) and incubated at 37°C for 18 hours with shaking. Following incubation, the culture was streaked onto MacConkey agar to verify bacterial enumeration. The final bacterial concentration used for the challenge was 1.2×10^{10} CFU/mL For the *E. coli* challenge treatments (NC, T1, and T2), pigs were orally inoculated with 10 mL of the bacterial suspension once daily for three consecutive days (days −2 to 0).

Composition and Microencapsulation of the Functional Additive

The functional additive complex (SynerBoost, Eugene Bio, Suwon, Korea) used in this study consisted of microencapsulated essential oils (thymol and carvacrol), coated organic acids (fumaric acid, citric acid, malic acid, and orthophosphoric acid), vitamin E (α-tocopherol), and sodium selenite. The encapsulation was performed using a hydrogenated palm oil matrix (particle size: 500–2,000 μm) via microencapsulation technology (Betagro, Italy), which enables slow-release of active compounds throughout the gastrointestinal tract.

Experimental design, animals, and housing

A total of 24 crossbred (Landrace × Yorkshire × Duroc) weaned pigs (12 barrows and 12 gilts) with an initial body weight of 9.61 ± 0.81 kg at 4 weeks of age were used in a feeding trial conducted for 17 days, consisting of an adaptation period of 3 days followed by an experimental period of 14 days. Pigs were randomly allotted to four dietary treatments in a completely randomized design with six replicates per treatment and one pig per replicate. The dietary treatments were as follows: 1) PC (basal diet without challenge); 2) NC (PC + *E. coli* challenge); 3) T1 (NC + 0.05% functional additive); and 4) T2 (NC + 0.10% functional additive). All experimental diets were formulated to meet the nutrient requirements recommended by NRC [15] (Table 1). Throughout the experimental

period, pigs had *ad libitum* access to feed and water. For the *E. coli* challenge treatments (NC, T1, and T2), pigs were orally inoculated with 10 mL of *E. coli* suspension for three consecutive days. Challenged pigs and non-challenged pigs were housed in separate rooms, and strict biosecurity procedures were implemented to prevent cross-contamination of *E. coli*.

Sampling and Analysis

Growth performance

BW was measured at the beginning of the experiment and subsequently on 7 and 14 days (end of the trial). Feed intake was calculated by subtracting the remaining feed from the amount provided at each BW measurement interval. ADG was determined by dividing the BW difference during each period by the number of days in that period. Feed efficiency was calculated as the ratio of ADG to average daily feed intake (ADFI).

Fecal score

Fecal scores were assessed for each pen within each treatment twice daily (08:00 and 17:00) by the same observer throughout the experimental period. Fecal were scored as follows: normal feces (0), soft feces (1), mild diarrhea (2), and severe diarrhea (3). The frequency of diarrhea over the entire experimental period was calculated as the number of days on which the average diarrhea score per pen was ≥ 2.

Nutrient digestibility

To evaluate nutrient digestibility, chromium oxide (Cr_2O_3) was added to the experimental diets at 0.4% on days 4 and 11 after the start of the experiment. Diet samples containing chromium oxide were also collected and stored at -20℃ alongside fecal samples until further analysis. Before analysis, fecal samples were dried in an oven at 60℃ for 72h and subsequently ground using a Wiley mill. The general nutrient composition and chromium concentrations of diets and feces were analyzed according to AOAC [16] procedures. Gross energy content was determined using an adiabatic bomb calorimeter (Model 6400, Parr Instrument, Moline, IL, USA). Nutrient digestibility was calculated using the following equation:

Digestibility (%) = {1 − (Cr_2O_3 concentration in diet, % × nutrient concentration in feces, %) / (Cr_2O_3 concentration in feces, % × nutrient concentration in diet, %)} × 100

Morphological analysis of small intestine

At the end of the experiment, approximately 10 cm of ileal tissue close to the ileocecal junction was excised for intestinal morphology analysis. The samples collected were rinsed with 10% formalin and fixed for 24 h. After fixation, the intestinal tissues were embedded in paraffin, sectioned at a thickness of 5 μm, and stained with hematoxylin and eosin. The prepared slides were examined using a light microscope (CX51, Olympus Optical Co., Ltd., Tokyo, Japan), and villus height (VH) and crypt depth (CD) were analyzed using Zen 3.4 software (Carl Zeiss Co. Ltd., NY, United States).

Expression of tight junction proteins

Total RNA was extracted from the collected samples using a Total RNA Extraction Kit (iNtRON Biotechnology, Seongnam, Korea). The extracted mRNA was converted to cDNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA, USA). Gene amplification was performed using Fast qPCR 2 × SYBR Green Master Mix (Applied Biosystems). PCR was conducted in two steps. The first step was an activation step performed at 95°C for 2 min for one cycle. The second step consisted of a denaturation step at 95°C for 15 s and an annealing/extension step at 54°C for 1 min, repeated for a total of 40 cycles. The target genes were mucin-1 (MUC1), zonula occludens-1 (ZO-1), claudin-1 (CLDN-1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primers used for amplification are shown in Table 2. Normalization was performed using the reference gene GAPDH. Relative gene expression was analyzed using the $2^{-\Delta\Delta}$Ct method.

Blood profiles

Blood samples were collected from the jugular vein of all pigs before the *Escherichia coli* challenge (day 0) and days 3 and 14 post-challenge. At each sampling time, blood samples were collected into vacuum tubes containing K3EDTA for complete blood count analysis and into non-heparinized tubes for serum analysis. After collecting, serum samples were centrifuged at 3,000 × g for 20 min at 4°C. The blood sample tubes were then stored at −20°C until further analysis. Serum concentrations of interleukin-8 (IL-8), interleukin-10 (IL-10), malondialdehyde (MDA), and interferon-γ were determined using ELISA kits (Quantikine, R&D Systems, Minneapolis, MN, USA), and absorbance was measured at 450 nm.

16S metagenomic data analysis

The 16S rRNA sequencing data were analyzed using the QIIME2 next-generation microbial community bioinformatics pipeline for metagenomic studies. Samples were sent to Sanigen (Anyang, Korea) for microbial

sequencing using 16S rRNA technology. All data were converted into QIIME2 artifacts, which include information on data type and source for downstream processing. Amplicon sequence variants (ASVs) were obtained from the sequence data using the Divisive Amplicon Denoising Algorithm 2 (DADA2) within the QIIME2 plugin, which detects and corrects amplicon errors and filters potential base errors and chimeric sequences. Relative taxonomic frequency tables indicating differential abundance testing at specific taxonomic levels were generated using the feature table and taxonomic collapse functions within QIIME2. The QIIME2 "diversity" plugin was used to estimate and plot alpha diversity metrics using R bioinformatics packages. This microbial diversity analysis pipeline was designed to use the ASV table from the ASV selection step as the required input data. Differences in species richness and evenness scores accounting for sampling depth were assessed using observed OTUs, Chao1, Shannon, and Simpson indices, which estimate the V3–V4 hypervariable regions of the bacterial 16S rRNA gene. In addition, differences in relative abundance were analyzed by comparing the mean bacterial proportions and compositions at each taxonomic rank. Taxonomic accuracy was further cross validated by comparing the concordance rates between ASV taxonomic assignments and NCBI bacterial taxonomy across different amplicon regions.

Statistical analysis

All data in this study, except for BW, ADG and fecal score, were analyzed utilizing JMP® Pro version 16.0.0 (SAS Institute Inc., Cary, NC, USA). A one-way analysis of variance (ANOVA) was conducted to evaluate differences among treatment groups, and the significance of treatment means was determined using Tukey's multiple range test to assess differences among treatment groups, with significance set at $p < 0.05$. BW and ADG were analyzed using an analysis of covariance (ANCOVA) model, with initial body weight used as a covariate. Fecal score was analyzed using the chi-square test.

RESULTS

Growth performance

The effects of dietary supplementation with functional additives (coated essential oils + coated organic acids + vitamin E and selenium) on the growth performance of weaned pigs are presented in Table 3. On day 7, the T1 and T2 showed significantly higher BW than the NC, and the T1 exhibited significantly higher BW than the NC on day 14 ($p < 0.05$). During days 0 to 7, the PC and T1 showed significantly higher ADG than the NC ($p < 0.05$). Throughout the overall experimental period, the T1 showed significantly higher ADG than the NC ($p < 0.05$).

Fecal score

The effects of dietary supplementation with functional additives on the fecal scores of weaned pigs are presented in Figure 1. During weeks 1 and 2, all treatment groups showed lower fecal scores than the NC ($p < 0.05$).

Nutrient digestibility

The effects of dietary supplementation with functional additives on the nutrient digestibility of weaned pigs are presented in Table 4. No significant differences were observed in nutrient digestibility (DM, CP, and GE) among treatments ($p > 0.05$).

Intestinal morphology

The effects of dietary supplementation with functional additives on the intestinal morphology of weaned pigs are presented in Table 5. In the ileum, VH was significantly higher in the PC than the NC ($p < 0.05$). CD was significantly lower in the PC and T1 than the NC ($p < 0.05$). The VH to CD ratio was significantly higher in the PC, T1, and T2 than the NC ($p < 0.05$).

Tight junction protein

The effects of dietary supplementation with functional additives on the tight junction protein of weaned pigs are presented in Table 6. The expression of CLDN-1 was significantly higher in the T1 and T2 than the NC and PC ($p < 0.05$). The expression of ZO-1 was significantly higher in the T1 than the NC ($p < 0.05$). The expression of MUC1 was significantly higher in the T1 and T2 than the NC and PC ($p < 0.05$).

Immune response

The effects of dietary supplementation with functional additives on the immune response of weaned pigs are presented in Table 7. On day 3, serum IL-8 concentration was significantly higher in the T2 than the PC ($p < 0.05$). On day 3, serum interferon-γ concentration was significantly higher in the T1 and T2 than the PC ($p < 0.05$).

Alpha diversity

The effects of dietary supplementation with functional additives on the alpha diversity of weaned pigs are presented in Table 8. No differences were observed in the alpha diversity parameters including Chao 1, Simpson, and Shannon indices on day 0 and 14 ($p > 0.05$).

Beta diversity

The effects of dietary supplementation with functional additives on the beta diversity of weaned pigs are presented in Figure 2-3. On day 0, unweighted UniFrac beta diversity showed a significant difference between the T1 and the NC ($p < 0.05$; Figure 2). Weighted UniFrac beta diversity also revealed significant differences between the PC and the T2, and between the T1 and the T2 ($p < 0.05$; Figure 2). On day 14, weighted UniFrac beta diversity showed significant differences between the PC and the T2, and between the T2 and the NC ($p < 0.05$; Figure 3).

Relative abundance

The effects of dietary supplementation with functional additives on the microbial composition of weaned pigs are presented in Figure 4-5. On day 0, at the genus and family levels, the T2 showed higher relative abundances of *Lactobacillus* and *Megasphaera* than the other treatment groups ($p < 0.05$; Figure 4). The NC showed lower relative abundances of *Phascolarctobacterium* and the *Christensenellaceae* R-7 group than the other treatment groups ($p < 0.05$; Figure 4). On day 14, the NC showed higher relative abundances of *Oscillosporaceae* and lower relative abundances of *Ruminococcus* than the other treatment groups. The PC showed lower relative abundances of *Parabacteroides* and higher relative abundances of *Prevotella* than other treatment groups ($p < 0.05$; Figure 5).

DISCUSSION

Pathogenic *E. coli* is the main cause of PWD, leading to intestinal damage and inflammatory responses that compromise barrier function and nutrient absorption [17]. The ETEC K88 strain used in this study possesses virulence factors including F4 fimbriae for intestinal adhesion and produces heat-labile toxin (LT) and heat-stable toxins (STa and STb), which disrupts epithelial barrier function and alters tight junction integrity, impairing intestinal epithelial function [18]. Epithelial injury weakens barrier integrity and increases intestinal permeability, allowing bacteria to translocate into the systemic circulation [18]. As a result, glucose and amino acids are mainly directed toward tissue repair and immune activation rather than growth, which reduces ADG [19]. This metabolic shift and reduced feed intake, caused by intestinal dysfunction, contribute to impaired nutrient utilization and decreased growth in pigs challenged with *E. coli* [20].

In the present study, the significant decrease in BW and ADG in the NC group, compared to the PC group, was attributed to the adverse effects of the *E. coli* challenge. However, the groups supplemented with functional additives showed improved BW and ADG, suggesting that EOs and OA can effectively mitigate these negative effects. Although these results should be interpreted with considering the relatively small sample size (n = 6 per treatment), which may have limited the statistical sensitivity to detect significant differences in growth performance parameters, the observed patterns are discussed in the context of the following mechanistic evidence. Thymol and carvacrol, the primary phenolic compounds in EOs, disrupt bacterial cell membrane permeability by altering the lipid bilayer structure [21]. This membrane disruption enhances the antimicrobial effect of OA, allowing it to penetrate bacterial cells and lower intracellular pH, which inhibits bacterial growth in the intestine [22]. This reduction in pathogenic bacterial populations enhances intestinal barrier function, thereby supporting BW and ADG [23]. Improved BW and ADG observed in the T1 group were accompanied by changes in ADFI, indicating that dietary supplementation influenced both intestinal health and feeding behavior [24]. However, previous studies have reported a quadratic increase in feed intake with EO supplementation at levels ranging from 250 to 1,000 mg/kg, with optimal feed intake observed at 500 mg/kg. This increase is attributed to the strong aroma of EOs, which reduces palatability at higher inclusion levels [25]. This aligns with the lower ADFI observed in the T2 group in the present study, suggesting that the higher supplementation level may have negatively impacted feed palatability.

Pathogenic *E. coli* disrupts the homeostasis of intestinal water and electrolytes by causing dysregulation of epithelial ion transport through enterotoxins [26]. *E. coli*-derived toxins elevate cyclic adenosine monophosphate

and cyclic guanosine monophosphate, promoting chloride secretion and inhibiting sodium absorption, leading to water loss and diarrhea [27]. Concurrently, *E. coli*-induced inflammation and TP disruption increase paracellular permeability, resulting in impaired fluid absorption and elevated fecal scores [28]. The reduction in diarrhea incidence observed with additive supplementation in this study suggests improved intestinal barrier function and a reduced pathogen load. Thymol and carvacrol permeabilize and depolarize the bacterial cytoplasmic membrane, leading to cell lysis [29]. OA in the blend lowers intestinal pH, creating adverse conditions for *E. coli* colonization [30]. Additionally, antioxidants like vitamin E and selenium help mitigate oxidative stress in enterocytes during bacterial challenges, preserving tight junction integrity and reducing paracellular water loss [11]. Overall, the antimicrobial activity and mitigation of oxidative stress provided by the additive complex reduced the fecal scores observed in this study.

Nutrient digestibility reflects nutrient utilization and absorption, and it is directly linked to pig growth performance. Park et al [31] reported that improvements in ATTD of DM and CP were accompanied by increased ADG in weaned pigs. However, in the present study, functional feed additives improved productivity without enhancing nutrient digestibility, which contrasts with previous findings. No significant differences were observed in the apparent total tract digestibility (ATTD) of DM, CP and GE among the treatment groups in this study. ATTD is calculated as the difference between nutrient intake and fecal nutrient output, which represents the apparent nutrient digestibility across the gastrointestinal tract without considering endogenous losses [32]. Therefore, ATTD values cannot distinguish between nutrient absorption in the small intestine and microbial fermentation in the large intestine, and they may not accurately reflect localized physiological changes in specific segments of the intestine [33]. This improvement can be attributed to the modulation of the gut microbiota, which reduces endogenous losses related to intestinal inflammation and microbial competition for nutrients [34]. The findings suggest that the beneficial effects of feed additives on growth performance may be attributed not only to improved nutrient digestibility but also to other mechanisms, such as the modulation of immune function or the maintenance of intestinal integrity.

E. coli challenge damages the intestinal epithelium, leading to enterocyte apoptosis and adaptive crypt cell hyperplasia, which manifest as decreased VH and increased CD [35]. In the present study, the groups supplemented with additives maintained significantly higher VH:CD ratios and lower CD compared to the NC group, indicating protective effects against intestinal damage induced by *E. coli*. These protective effects may be attributed to several distinct mechanisms. EO exhibit antimicrobial activity against pathogenic *E. coli*, and this selective antimicrobial action reduces the pathogen load in the gut [36]. Additionally, the OA (formic acid and

citric acid) in the formulation reduce intestinal pH to inhibit pathogenic bacteria growth while supporting beneficial microbiota [37]. Furthermore, vitamin E and selenium act as antioxidants that reduce oxidative stress caused by *E. coli* infection, protecting enterocytes from oxidative damage and supporting the integrity of the intestinal barrier [13]. Collectively, EO, OA, and antioxidants preserved intestinal morphology and prevented the crypt hyperplasia typically observed in weaned pigs challenged with *E. coli*. Collectively, EO, OA, and antioxidants preserved intestinal morphology and prevented the crypt hyperplasia typically observed in weaned pigs challenged with *E. coli*. These improvements in intestinal barrier function, rather than changes in nutrient digestibility, may have been the primary mechanism contributing to the enhanced growth performance observed in the additive-supplemented groups.

The maintenance of intestinal barrier integrity was demonstrated by the upregulation of barrier-related genes in the ileal mucosa [38]. The current study showed that additive complexes significantly increased CLDN-1 and ZO-1 mRNA expression, indicating enhanced tight junction assembly and improved epithelial barrier function. Tight junctions form the primary seal between adjacent epithelial cells, and their upregulation directly contributes to reduced paracellular permeability and decreased bacterial translocation [39]. MUC1 acts as the first line of defense in the intestinal mucosa by creating a protective mucus layer that prevents direct contact between pathogens and epithelial cells [40]. The simultaneous enhancement of both tight junctions and the mucus layer suggests a comprehensive restoration of the intestinal defense system [41]. Consistent with previous studies, this result indicated that the additive complex not only reduced pathogen burden but also promoted the maintenance of epithelial barrier function.

Immune markers IL-8 and IFN-γ are essential for evaluating the immune response in weaned pigs [42]. IL-8 is a pro-inflammatory cytokine that acts as a strong chemoattractant for neutrophils, enhancing early innate immune defense by guiding immune cells to sites of infection [43]. IFN-γ, a key Th1 cytokine, promotes macrophage activation and bolsters cellular immunity against intracellular pathogens [44]. Both IL-8 and IFN-γ levels rise rapidly in response to *E. coli* infection during the acute inflammatory phase, then decrease during the recovery phase as the immune response subsides [45]. In this study, *E. coli* challenge led to a significant increase in IL-8 and IFN-γ concentrations on day 3 in the NC group compared to the PC group, with levels returning to baseline in all groups by day 14. These temporal changes align with previous findings, which reported heightened inflammatory markers at early time points followed by a decline later on [46]. Notably, IL-8 and IFN-γ concentrations were numerically higher in the additive-supplemented groups on day 3, however returned to baseline by day 14 similar to the other groups. This transient elevation reflects enhanced early immune activation

rather than inflammatory stress, as the rapid resolution indicates a controlled immune response against *E. coli* infection [47]. The functional additive containing vitamin E and selenium enhanced early responses of IFN-γ and IL-8 by supporting T cell-mediated Th1 activation and maintaining effective inflammatory signaling [48]. This study suggests that the additive enhanced immune activation during the acute phase of infection while ensuring an appropriate resolution of the response without excessive inflammation.

Alpha diversity measures species richness and evenness within individual samples, while beta diversity compares microbial community composition between samples [49]. In this study, alpha diversity did not show significant differences among treatments; however, distinct clustering in beta diversity indicated that the additive modified microbial composition through qualitative shifts rather than quantitative changes [50]. The absence of significant differences in alpha diversity, despite distinct beta diversity clustering, suggests that the additive selectively shifted microbial composition toward functionally beneficial taxa without reducing overall species richness, potentially supporting a more stable intestinal ecosystem in weaned pigs. These compositional shifts were reflected in the relative abundance of individual bacterial taxa linked to intestinal function. The increased abundance of *Lactobacillus* in the additive-supplemented groups indicates enhanced pathogen inhibition through competitive exclusion and antimicrobial metabolite production [51]. *Lactobacillus* competes with pathogenic *E. coli* for adhesion sites and nutrients while creating an acidic environment unfavorable for pathogens [52]. Additionally, *Megasphaera* converts lactate produced by *Lactobacillus* into butyrate and propionate, which serve as energy sources for colonocytes and possess anti-inflammatory properties [53]. The metabolic cooperation between *Lactobacillus* and *Megasphaera* improves fermentation stability and prevents lactate accumulation [54]. These microbial shifts may have mechanistically contributed to the upregulation of tight junction proteins, including CLDN-1 and ZO-1, as butyrate produced by Megasphaera has been shown to enhance tight junction assembly and reduce epithelial permeability [55]. Furthermore, the increased abundance of Lactobacillus may have modulated immune responses by promoting Th1 activation and regulating pro-inflammatory cytokine signaling, which aligns with the observed changes in IL-8 and IFN-γ concentrations in the present study [56]. Changes in the abundance of *Ruminococcus* and *Oscillosporaceae* indicate shifts in fiber degradation and microbial balance [57]. *Ruminococcus* breaks down complex polysaccharides and produces acetates that support other beneficial bacteria, while *Oscillosporaceae* contributes to short-chain fatty acid production and community stability [58]. These findings suggest that the additive shifted gut microbial composition toward a more favorable balance, potentially enhancing gut health and nutrient utilization.

Conclusion

E. coli challenge impaired growth performance and intestinal health in weaned pigs. Dietary supplementation with a coated complex additive containing essential oils, organic acids, vitamin E, and selenium effectively mitigated these negative effects. Both supplementation levels (0.05% and 0.10%) improved body weight, intestinal morphology, and barrier function and decreased diarrhea incidence, while 0.05% supplementation showed the greatest improvement in average daily gain, and 0.10% supplementation was associated with shifts in gut microbial composition. These results suggest that 0.05–0.10% supplementation of this encapsulated complex additive supports intestinal health in weaned pigs under *E. coli* challenge.

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TABLES AND FIGURES

Table 1. Compositions of basal diets (as-fed-basis)

Items	Content
Ingredients, %	
Corn	34.43
Extruded corn	15.00
Lactose	10.00
Dehulled soybean meal, 51% CP[1]	13.50
Soy protein concentrate, 65% CP[1]	10.00
Plasma powder	6.00
Whey	5.00
Soy oil	2.20
Monocalcium phosphate	1.26
Limestone	1.40
L-Lysine-HCl, 78%	0.06
DL-Methionine, 50%	0.15
Choline chloride, 25%	0.10
Vitamin premix[2]	0.25
Trace mineral premix[3]	0.25
Salt	0.40
Total	100.00
Calculated value[4]	
ME, Kcal/kg	3433
CP, %	20.76
Lysine, %	1.35
Methionine, %	0.39
Ca	0.82
P	0.65
Analyzed value	
ME, kcal/kg	3512
CP, %	20.92

[1] CP, crude protein.
[2] Provided per kg of complete diet: vitamin A, 11,025IU; vitamin D_3, 1103IU; vitamin E, 44 IU; vitamin K, 4.4mg; riboflavin, 8.3mg; niacin, 50mg; thiamine, 4mg; d-pantothenic, 29mg; choline, 166mg; and vitamin B_{12}, 33mg.
[3] Provided per kg of complete diet without Zinc: Cu (as $CuSO_4 \cdot 5H_2O$), 12mg; Mn (as MnO_2), 8mg; I (as KI), 0.28mg; and Se (as $Na_2SeO_3 \cdot 5H_2O$), 0.15mg.
[4] Values were calculated using National Swine Nutrition Guide (NSNG; V 2.0).

Table 2. Primer sequences used for the RT-qPCR analysis with the Mucin1, ZO-1, CLDN1, and GAPDH genes

Gene	Primers	Sequence (5'-3')
Glyceraldehyde-3-phosphate dehydrogenase 2 (GAPDH)	Forward	TCGGAGTGAACGGATTTGGC
	Reverse	TGACAAGCTTCCCGTTCTCC
Mucin 1	Forward	CCACAACCTGAAGACACAGT
	Reverse	GACCAGAATACAGACCAGCA
ZO-1	Forward	CTCTGTCCATGCAGATAAGC
	Reverse	AATAGCTCCCTGTGGGATAA
CLDN1	Forward	GCTGGGACTAATAGCCATCT
	Reverse	AAGAGAGCCTGACCAAATTC

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Table 3. Effects of feed additive complex on growth performance in weaned piglets

Item	NC	PC	T1	T2	SE	*P*-value
BW, kg						
D-3	9.62	9.60	9.62	9.62	0.450	1.000
D 0	10.11	10.12	10.15	10.14	0.443	0.925
D 7	11.26b	11.67ab	11.74a	11.56a	0.113	0.013
D 14	12.92b	13.58ab	13.65a	13.42ab	0.264	0.026
ADG, g						
D-3 to 0	126.88	133.75	135.00	138.13	17.187	0.972
D 0 to 7	164.63b	221.28a	226.64a	202.43ab	12.737	0.011
D 7 to 14	236.08	272.72	273.27	266.26	16.249	0.389
D 0 to 14	200.36b	247.00ab	249.96a	234.35ab	12.322	0.039
ADFI, g						
D-4 to 0	194.38	193.75	199.38	201.11	5.774	0.755
D 0 to 7	298.93b	352.14a	352.86a	321.79b	7.188	<0.001
D 7 to 14	413.57	409.29	416.07	415.71	7.528	0.914
D 0 to 14	356.25	380.71	384.46	368.75	7.958	0.083
G:F, g/g						
D-3 to 0	0.65	0.69	0.68	0.69	0.086	0.989
D 0 to 7	0.60	0.67	0.65	0.63	0.044	0.748
D 7 to 14	0.64	0.69	0.66	0.64	0.039	0.779
D 0 to 14	0.62	0.68	0.65	0.64	0.038	0.713

NC (PC + *E. coli* challenge); PC, basal diet; T1 (NC + feed additive complex 0.05%), T2 (NC + feed additive complex 0.10%); BW, body weight; ADG, average daily gain; ADFI, average daily feed intake; G:F, feed efficiency; SE, standard error

Table 4. Effects of feed additive complex on nutrient digestibility in weaned piglets

Item, %	NC	PC	T1	T2	SE	*P*-value
D 7						
DM	80.56	79.77	80.42	80.08	0.657	0.829
CP	72.17	71.19	73.42	73.32	0.872	0.374
GE	81.77	81.47	82.21	81.32	0.584	0.716
D 14						
DM	79.38	79.37	80.18	80.07	0.781	0.816
CP	72.59	71.42	72.94	72.96	1.151	0.759
GE	80.99	81.11	81.95	81.10	0.839	0.840

NC (PC + *E. coli* challenge); PC, basal diet; T1 (NC + feed additive complex 0.05%), T2 (NC + feed additive complex 0.10%); DM, dry matter; CP, crude protein; GE, gross energy; SE, standard error

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Table 5. Effects of feed additive complex on intestinal morphology in weaned piglets

Item	NC	PC	T1	T2	SE	*P*-value
Villus						
Height, μm	318.02b	389.34a	356.90ab	360.09ab	11.025	0.002
Width, μm	108.00	108.50	106.50	107.00	3.500	0.667
Density, NO./mm^2	12.00	13.00	13.00	12.00	0.405	0.982
Crypt						
Depth, μm	200.40a	172.80b	181.99b	184.03ab	4.265	0.002
Width, μm	169.00	167.00	170.00	169.00	7.000	0.561
Density, NO./mm^2	24.00	25.00	24.00	25.00	0.689	0.917
VH:CD ratio	1.59b	2.27a	1.96a	1.96a	0.087	<0.001

NC (PC + *E. coli* challenge); PC, basal diet; T1 (NC + feed additive complex 0.05%), T2 (NC + feed additive complex 0.10%); VH:CD, Villus height: Crypt depth, SE, standard error

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Table 6. Effects of feed additive complex on tight junction in weaned piglets

Item	NC	PC	T1	T2	SE	*P*-value
CLDN-1	0.79b	1.00b	1.37a	1.25a	0.047	<0.001
ZO-1	0.72b	1.00ab	1.47a	1.23ab	0.119	0.012
MUC1	0.81b	1.00b	1.29a	1.37a	0.048	<0.001

NC (PC + *E. coli* challenge); PC, basal diet; T1 (NC + feed additive complex 0.05%), T2 (NC + feed additive complex 0.10%); CLDN-1, claudin-1; ZO-1, zonula occuldins-1; MUC1, mucin1; SE, standard error

527

Table 7. Effects of feed additive complex on immune response in weaned piglets

Item	NC	PC	T1	T2	SE	*P*-value
D 0						
IL-8	2144.29	2123.67	2217.73	2142.08	173.922	0.981
IL-10	43.68	45.43	47.90	43.87	2.086	0.481
MDA	70.77	72.89	76.95	71.42	11.397	0.980
IFN-γ	190.57	187.51	185.66	188.64	13.124	0.995
D 3						
IL-8	2481.93ab	1785.84b	2719.43ab	2937.39a	254.629	0.039
IL-10	49.90	46.33	61.48	62.88	7.026	0.295
MDA	123.71	74.60	122.29	119.92	22.176	0.370
IFN-γ	267.16ab	202.17b	314.15a	301.46a	19.974	0.008
D 14						
IL-8	1915.27	2090.19	2009.03	2370.69	140.289	0.174
IL-10	51.67	48.63	48.51	49.88	1.718	0.553
MDA	84.19	67.37	71.41	73.25	21.761	0.953
IFN-γ	183.84	155.29	180.52	174.27	10.397	0.263

NC (PC + *E. coli* challenge); PC, basal diet; T1 (NC + feed additive complex 0.05%), T2 (NC + feed additive complex 0.10%); IL, interleukin; MDA, malondialdehyde; IFN, interferon; SE, standard error

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Table 8. Effects of feed additive complex on alpha diversity in weaned piglets

Item	NC	PC	T1	T2	SE	*P*-value
D 0						
Chao 1	910.40	895.30	940.22	955.18	50.600	0.438
Shannon	7.55	7.71	7.90	7.55	0.169	0.483
Simpson	0.98	0.99	0.99	0.98	0.002	0.317
D 14						
Chao 1	967.50	906.00	1066.55	1158.13	58.950	0.068
Shannon	7.19	7.39	7.66	7.67	0.121	0.064
Simpson	0.98	0.99	0.99	0.99	0.001	0.225

NC (PC + *E. coli* challenge); PC, basal diet; T1 (NC + feed additive complex 0.05%), T2 (NC + feed additive complex 0.10%); SE, standard error

529
530

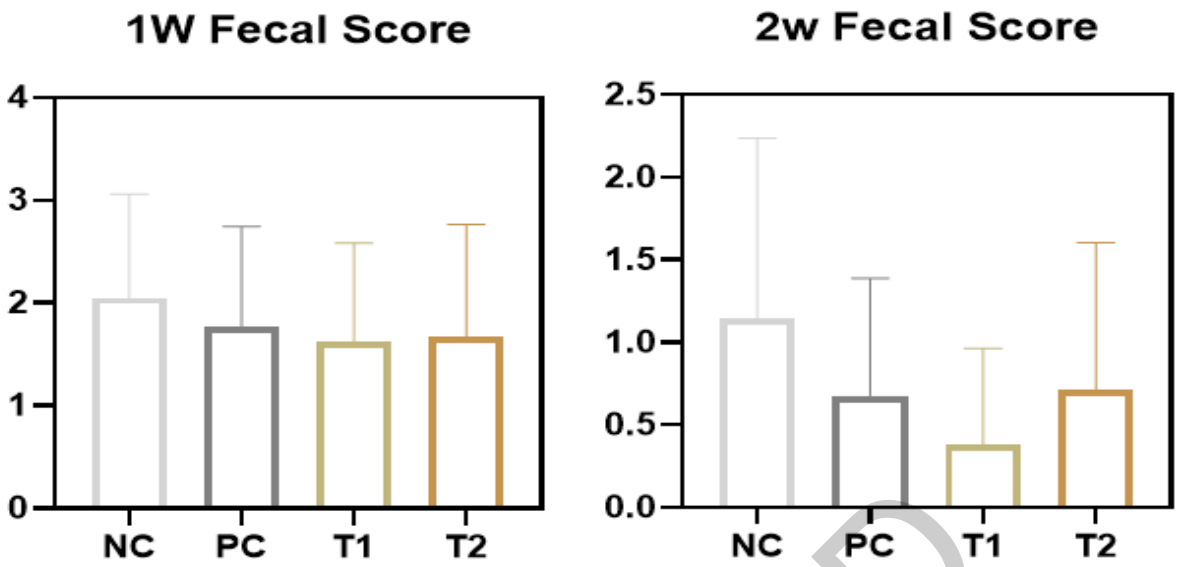


Figure 1. Effects of feed additive complex on fecal score in weaned piglets**.** NC (PC + *E. coli* challenge); PC, basal diet; T1 (NC + feed additive complex 0.05%), T2 (NC + feed additive complex 0.10%); χ^2= 20.640, p=0.014 (D 7 fecal score); χ^2= 22.342, p=0.008 (D 14 fecal score)

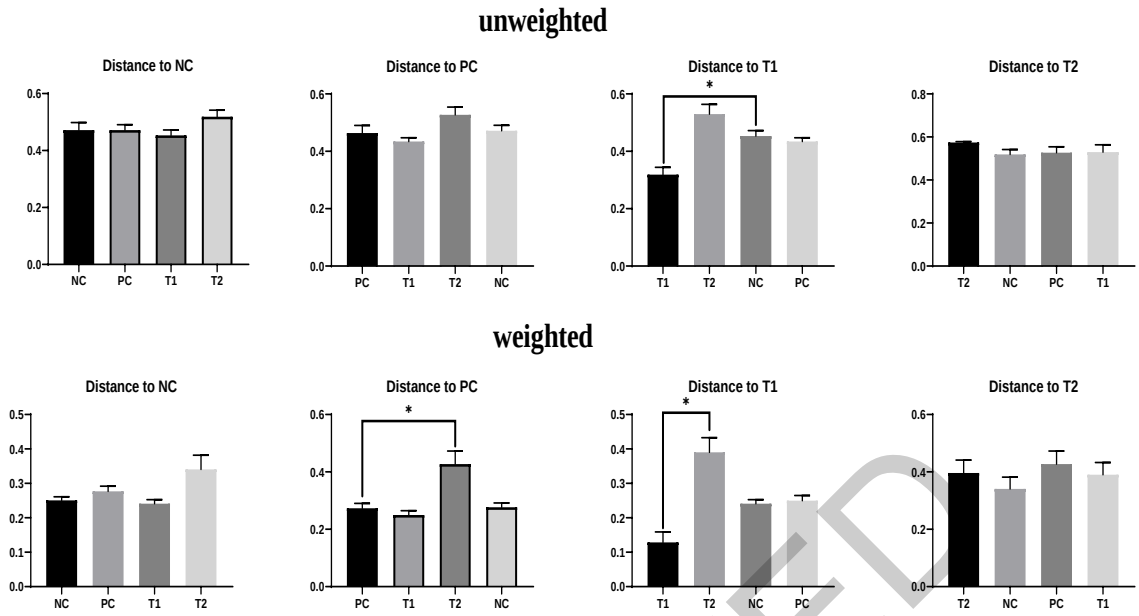
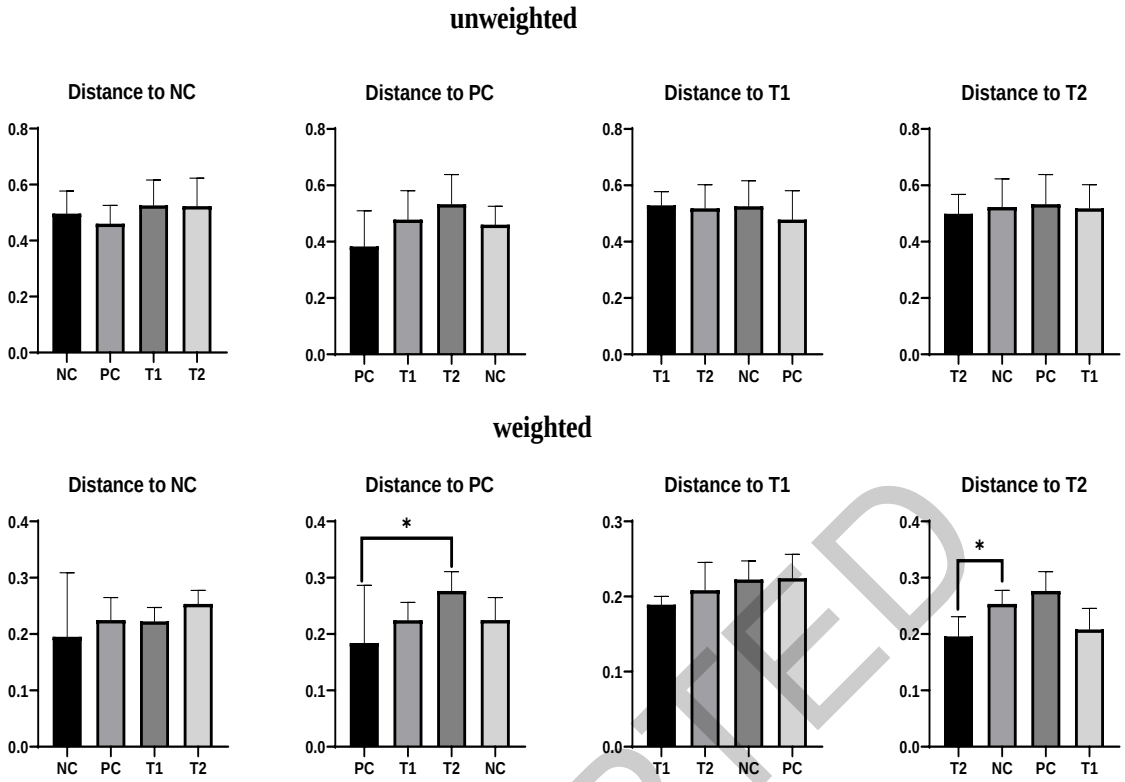


Figure 2. Effects of feed additive complex on b-diversity in weaned piglets on Day 0. NC, PC + *E. coli* challenge; PC, basal diet; T1, NC + feed additive complex 0.05%; T2 NC + feed additive complex 0.10%



541

542 **Figure 3.** Effects of feed additive complex on b-diversity in weaned piglets on D 14**.** NC, PC + *E. coli*
543 challenge; PC, basal diet; T1, NC + feed additive complex 0.05%; T2 NC + feed additive complex 0.10%

544

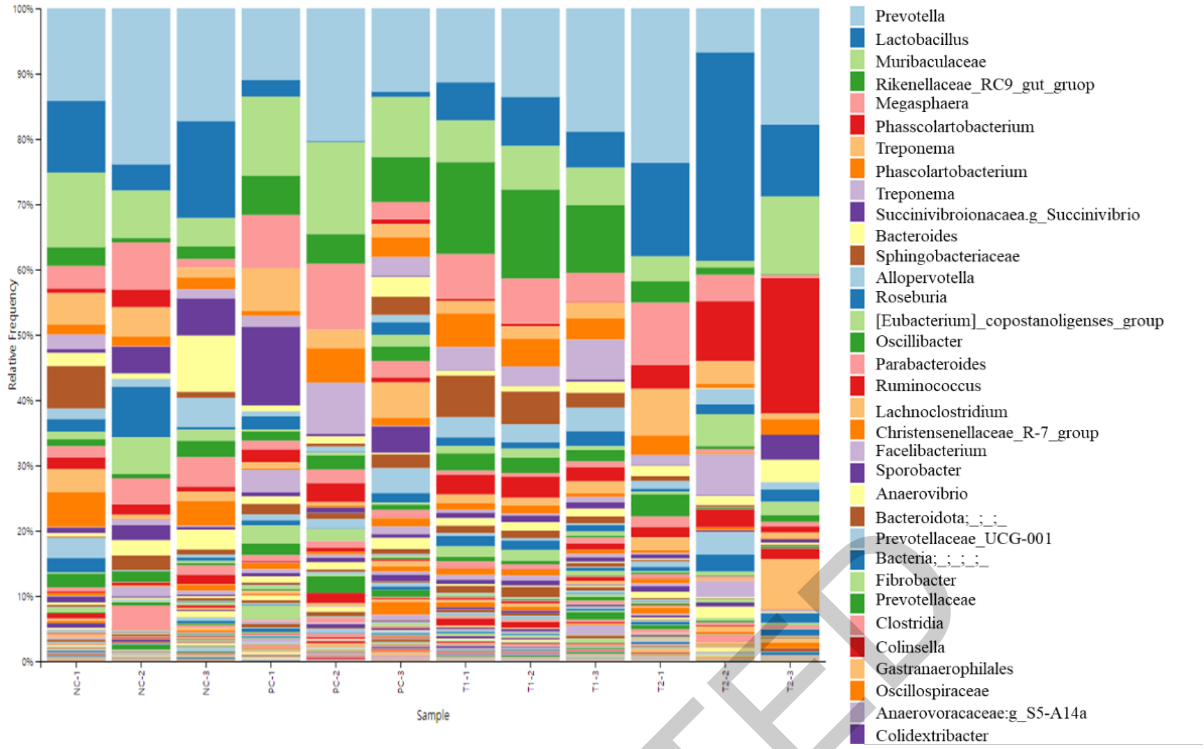


Figure 4. Effects of feed additive complex on relative abundance in weaned piglets on D 0. NC, PC + *E. coli* challenge; PC, basal diet; T1, NC + feed additive complex 0.05%; T2 NC + feed additive complex 0.10%

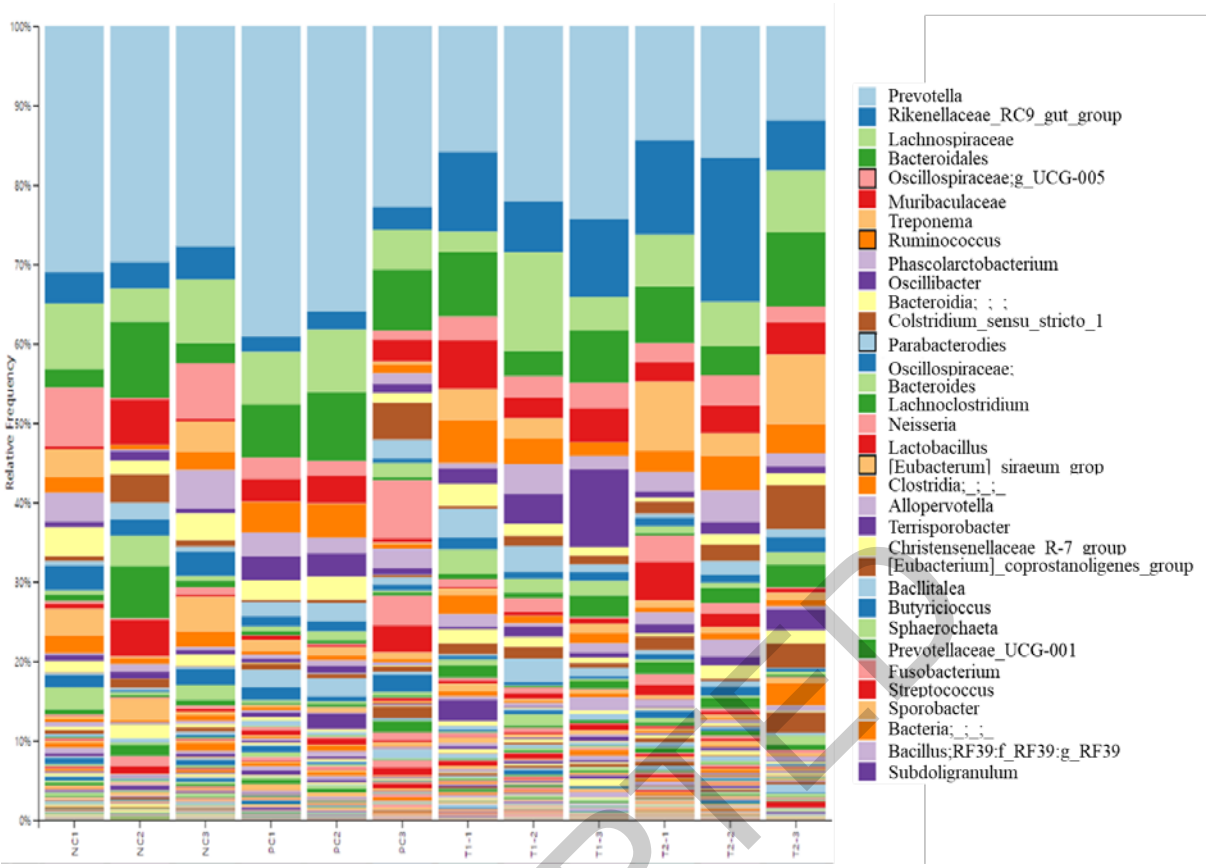


Figure 5. Effects of feed additive complex on relative abundance in weaned piglets on D 14. NC, PC + *E. coli* challenge; PC, basal diet; T1, NC + feed additive complex 0.05%; T2 NC + feed additive complex 0.10%