RESEARCH REPORT
PORK SAFETY

Title: Comparison of the effect of direct-fed microbials and antibiotic supplementation on the growth response of weanling pigs.
NPB #03-028

Investigator: Dr. Charles Maxwell

Institution: University of Arkansas, Fayetteville

Co-Investigators: Dr. Tom Rehberger, Agtech Products, Inc., Waukesha, WI
Dr. Ellen Davis; University of Arkansas, Fayetteville

Date Received: January 31, 2005

Abstract: Pigs (n=252) from 30 litters were used to compare the effect of direct-fed microbials and antibiotic supplementation on the growth of nursery pigs. Beginning at farrowing, pigs were provided milk supplementation throughout lactation with or without the addition of *Lactobacillus brevis* (1E1) via an in-line system. These treatments were continued during the nursery period, in which pigs that were administered 1E1 in lactation continued to receive 1E1 through the watering system. At the start of the nursery phase, pigs were fed a control basal diet devoid of *Bacillus* or antibiotics, the basal diet with *Bacillus* cultures, or the basal diet with antibiotic supplementation. These dietary treatments were administered during Phase 1 (d 0 to 14), Phase 2 (d 14 to 28), and Phase 3 (d 28 to 38) post-weaning, in a 2 x 3 factorial design during the nursery period. On d 10, 20, and 38 after weaning, 12 pigs were euthanized, and gastrointestinal tissue sections were obtained for the enumeration of coliforms and *E. coli*, denaturing gradient gel electrophoresis (DGGE) of the GI microbial community DNA, and for villus, crypt, and goblet cell morphology measurements. Prior to euthanization a blood sample was collected for analysis of blood monocyte/macrophage phagocytosis. Data were analyzed using the GLM procedure of SAS, and the effects of 1E1 supplementation, dietary treatments, and their interaction were evaluated. No interactions were observed between 1E1supplementation and the dietary treatments ($P > 0.15$) for growth performance measurements. Pigs supplemented with 1E1 had greater ADG ($P < 0.05$) during Phase 2 and the overall nursery period (d 0 to 38), greater ADFI ($P < 0.05$) during Phase 3 and the overall nursery period, but tended to have lower gain:feed ($P < 0.10$) during Phase 3 of the nursery period. Pigs fed 1E1 were 1.58 kg heavier at the end of the nursery period than those fed milk replacer without 1E1 ($P < 0.01$). Pigs supplemented with antibiotics during the nursery had greater ($P < 0.01$) ADG during Phase 2 and the overall nursery period, and greater ($P < 0.01$) ADFI during Phase 3 than pigs fed the basal diet or pigs fed *Bacillus*.

Although pigs supplemented with antibiotics had greater ($P < 0.05$) gain:feed than pigs fed *Bacillus* during Phase 2, pigs fed *Bacillus* had greater ($P < 0.01$) gain:feed during Phase 3 than pigs fed the basal diet or those fed antibiotics. In the duodenum, pigs administered 1E1 had greater ($P < 0.05$) coliform counts on d 10 after weaning compared to pigs that did not receive 1E1, however, coliform counts were highest on d 20after weaning in pigs that did not receive 1E1 and tended to be greater ($P = 0.10$) than coliform counts from pigs administered 1E1 (1E1 x day interaction, $P < 0.05$). In the jejunum, the greatest adjusted *E. coli* counts were observed on d 20 after weaning in pigs not provided with 1E1, and these counts were significantly greater ($P < 0.05$) than those observed on d 20 after weaning when pigs were administered 1E1. The administration of 1E1 did not affect adjusted *E. coli* counts within the jejunum on d 10 or d 38 after weaning (1E1 x day interaction, $P < 0.05$). Pigs provided 1E1 had a lower ($P < 0.05$) villus height:crypt depth ratio in the duodenum and the jejunum on d 38 after weaning compared to pigs not administered 1E1, whereas there was no difference in the villus:crypt ratio between the 1E1 treatments on d 10 and d 20 after weaning (1E1 x day interaction, $P < 0.01$). In the ileum, villus height was greater ($P < 0.05$) in pigs fed the antibiotic diet compared to pigs fed the control diet or *Bacillus* on d 10 after weaning, however, pigs fed *Bacillus* exhibited greater ($P < 0.05$) villus height within the ileum on d 38 after weaning compared to the other dietary treatments (nursery diet x day interaction, $P < 0.01$). In the duodenum, the administration of 1E1 decreased ($P < 0.05$) the number of sulfated goblet cells while increasing ($P < 0.05$) the number of acidic goblet cells, compared to pigs that did not receive 1E1. Pigs fed diets containing *Bacillus* and antibiotic had a lower ($P < 0.05$) number of sulfated goblet cells in the duodenum than pigs fed the control diet during the nursery period. Also, acidic goblet cells were more abundant in the jejunum of pigs administered 1E1 compared to pigs not provided 1E1, whereas sulfated goblet cells were less abundant in the jejunum when pigs were provided with 1E1. Pigs fed the control diet and those fed *Bacillus* had a greater ($P < 0.09$) percentage of phagocytic monocyte/macrophages than pigs fed the antibiotic diet in the absence of 1E1 administration. However, with 1E1 administration, pigs fed *Bacillus* had fewer phagocytic monocyte/macrophages than pigs fed the control diet (1E1 x diet interaction, $P < 0.10$). On d 10 after weaning, pigs fed the *Bacillus* diet in the absence of 1E1 had a higher ($P < 0.05$) proportion of $CD4^+CD8^-$ lymphocytes within the $CD25^+$ population than pigs fed the control diet without 1E1. Although experimental treatments did not alter ($P > 0.05$) this cell population on d 20 after weaning, the proportion of $CD4^+CD8^-$ within the $CD25^+$ population was higher ($P < 0.05$) in pigs fed *Bacillus* in the presence of 1E1 compared to pigs fed *Bacillus* and antibiotic in the absence of 1E1 and the control and antibiotic diets in the presence of 1E1 (1E1 x diet x day interaction, $P = 0.04$; Figure 18). This study indicates that direct-fed microbial supplementation improves the growth performance of pigs during the nursery period, and results in potentially beneficial alterations in gastrointestinal microflora, morphology, monocyte/macrophage activity, and immune cell populations.

Key Words: Swine, Lactobacillus, Feed Supplements

Introduction: There has been increasing pressure on the livestock industry to decrease or discontinue the use of antibiotics in animal agriculture because of the risk of increasing the incidence of antibiotic-resistant bacteria. Several studies have been conducted to assess the presence of antibiotic-resistant bacteria within swine herds (Dawson et al., 1984; Matthew et al., 1998; van Den Boggard et al., 2002) and the Danish Integrated Antimicrobial Resistance Monitoring and Research Program recently reported that the discontinuance of specific antibiotics in swine herds led to a decrease

in bacteria resistant to those antibiotics (DANMAP, 2002). The efficacy of antimicrobial supplementation in improving growth and efficiency in swine is well documented, and the magnitude of improvement from antibiotic addition is greater in young pigs when compared to those in the later growing stages (Cromwell, 2001). The greater response of young pigs to antibiotic supplementation is a consequence of increased enteric pathogenic challenges as a result of the abrupt change from a diet of sow's milk to solid feed, as well as a change in the environment that accompanies the weaning process. In addition, the young piglet's immune system is not fully equipped to deal with pathogenic challenges.

The gastrointestinal tract of the pig harbors a metabolically active microbiota that stimulate the normal maturation of host tissues and provide key defenses that are not present in gnotobiotic animals (Gaskins, 2001). The gastrointestinal tract of the unborn fetus is sterile under normal conditions and is progressively colonized by microbial organisms starting with the birth process and continuing until the "normal" microbiota of the adult gastrointestinal tract develops (Mackie et al., 1999). The establishment of the intestinal microflora is adversely altered during weaning resulting in increased populations of potentially pathogenic acid-intolerant coliforms and a decline of favorable lactobacilli (Bolduan, 1999). These microbes have an immense influence on nutritional, physiological, and immunological processes in the host animal (Zoetendal, 2004b). Although a relatively stable microbiota eventually establishes in the mature pig's digestive tract, it requires a considerable amount of adaptation during its early life periods.

Traditional attempts to characterize the GI microflora have relied upon culturing techniques. The current estimation of GI microflora cultivability is 10-50%, making the unculturable bacteria the majority (Zoetendal, 2004a). To obtain more comprehensive views of microbial communities, culture independent techniques have been employed. Microbial community structure, diversity, and phylogeny can be directly analyzed by performing 16S ribosomal RNA (rRNA) sequence analysis. The 16S rRNA gene is ideal for culture independent comparisons because it is ubiquitous, it has a conserved function, the gene is easy to sequence, and there exists a large database of 16S rRNA sequences available for sequence alignment and comparison. This segment of DNA has both conserved and variable regions, making it an ideal target for PCR and subsequent comparisons. Denaturing gradient gel electrophoresis (DGGE) was first used for bacterial community analysis in 1993 by Muyzer et al. DGGE allows for the separation of similarly sized segments of DNA (PCR products) based upon nucleotide content, creating a community profile. When used in conjunction with 16S rDNA primers, DGGE can be used to determine the microbial makeup of complex communities. For example, DGGE has successfully been used to study the GI tract microflora for humans, pigs, cattle, dogs, rodents, and chickens (Zoetendal, 2004b).

The mechanisms by which antibiotics improve pig performance have not been completely elucidated. One hypothesis is that alterations in the intestinal microbial population may increase the production of positive growth factors such as insulin-like growth factor (IGF)-1 (Hathaway et al., 1999), as well as decrease the production of negative growth factors such as the inflammatory cytokines, which have been documented to decrease feed intake and negatively alter metabolic growth processes (Spurlock, 1997). The effects of nutritional additives on improved performance in the young pig is consistent with the hypothesis that apparent effects of luminal nutrients on the intestinal immune system are instead mediated through microbial shifts in response to exogenous nutrient availability (Gaskins, 2001).

The need for alternatives to antibiotic supplementation has prompted scientists to explore additional methods to improve pig growth and health. Bacteria of the genus

Lactobacillus are normal inhabitants of the gastrointestinal tract of many animal species, and in pigs, they are one of the principle bacterial groups in the proximal region of the digestive tract (Barrow et al., 1977). Genetic analysis of isolates from the gastrointestinal tracts of healthy and sick pigs in our laboratory indicated that the lactobacilli populations in healthy pigs were more homogenous than populations in sick pigs. The predominant isolate in healthy pigs represented 59% of the total lactobacilli and was identified as strain 1E1 (Parrott et al., 1994). In an additional study, total coliforms and *E. coli* were reduced by 2-3 logs by the inclusion of strain 1E1 during lactation (Banach et al., 2002). An additional direct-fed microbial containing *Bacillus* strains having inhibitory effects against *E. coli* serotypes F18 and K88 was selected following the screening of isolates native to infected swine herds (Parrott, et al., 2001).

The administration of direct-fed microbials such as lactobacilli and *Bacillus* strains, may be a more direct approach to alter the intestinal microflora and decrease populations of bacteria having a negative impact on pig performance than antibiotic supplementation. Directly altering the microbial colonization of the intestinal tract could be a means to improve swine growth and efficiency, by reducing the level of specific pathogens while establishing a more beneficial microbial population. This could decrease or eliminate the industry's reliance on growth-promoting levels of antibiotics. In this paper, we describe the use of expertise developed in our laboratory to evaluate the ability of specifically selected direct-fed microbials to beneficially alter the intestinal microflora and potentiate immune responsiveness.

Objectives:

1) Evaluate the impact of antibiotics vs. specifically selected direct-fed microbials administered to piglets during lactation and the nursery phase of production.
2) Evaluate the alterations in intestinal microbial populations in response to the administration of specifically selected direct-fed microbials vs. antibiotics to piglets during lactation and during the nursery phase of production.
3) Determine the effect of specifically selected direct-fed microbials vs. antibiotics on gastrointestinal and systemic immune responses of piglets during lactation and during the nursery period.

Materials and Methods:

Animals, Housing and Environment: Thirty litters from one farrowing group were administered milk supplementation with and without the direct-fed microbial supplement *Lactobacillus brevis* 1E1 via a liquid feeding system in addition to sow's milk during the lactation period. Pigs were farrowed in an environmentally controlled farrowing room containing 30 crates, each equipped with an in-line milk supplement system. Milk supplement containing 1E1 (5×10^9 cfu/pig/day) and devoid of antibiotics was administered to one half of the litters within the farrowing group. The remaining litters were administered milk supplement devoid of 1E1 and antibiotics. Milk supplement was administered ad libitum in a small bowl supplied by two 30-gallon tanks. Each tank was equipped with a hydro pump with a pressure regulator that pumped the milk supplement to the pens as needed. A bowl with a baby pig nipple was available in each crate, allowing milk to flow only when touched by a pig's nose to minimize spillage and waste of milk supplement. On a daily basis the entire system was flushed with hot water to remove spoiled milk or sediment, and fresh milk was prepared using a commercial milk supplement (Litter-Gro, Merrick's, Inc. Union Center, WI).

Following the lactation phase, a total of 252 pigs from 30 litters (126 from each lactation treatment) were blocked based on body weight in a 2 x 3 factorial arrangement of treatments (three dietary treatments with and without 1E1 supplementation) and were

moved to a wean-to-finish facility. Pigs were housed 7 pigs/ pen in totally slatted pens (1.52m x 3.05 m) equipped with radiant heaters, a two hole nursery feeder and wean-to-finish cup waterers. Ambient room temperature was maintained at approximately 78°F. In addition, a radiant heater provided supplemental heat to a 6' diameter covering two pens/heater during the nursery phase. Pigs administered milk supplement with and without the 1E1 microbial supplement prior to weaning were maintained on their same treatment through the nursery period by administering the 1E1 supplement (5×10^8 cfu/pig/day) through the watering system. Water was available throughout the study.

Diets: Three dietary treatments were fed during the nursery phase: 1) a control diet devoid of antibiotic or *Bacillus*, 2) *Bacillus* (7.5×10^5 cfu/g feed) administered in the control diet, and 3) the control diet supplemented with the antibiotic, Carbadox (Table 1-3). Treatments were administered throughout the nursery period (0 to 38 d after weaning). On the day of weaning, pigs were offered ad libitum access to Phase 1 nursery diets from day 0 to 14 after weaning, Phase 2 diets from day 14 to 28, and Phase 3 diets from day 28 to 38. Pigs allotted to treatments including *Bacillus* and 1E1 were separated from those fed diets devoid of the microbial to eliminate any exposure to the feces of the microbial-fed pigs. All diets met or exceeded NRC (1998) requirements for all nutrients. The experiment was carried out in accordance with the Animal Care Protocol # 01015 for swine experiments issued by the University of Arkansas Animal Care and Use Committee.

Data Collection: Pig BW and supplemental milk intake were determined at 5, 10, 14, and 21 days of age and at weaning during the lactation phase. During the nursery phase, pig BW and feed intake were measured at the end of each phase. Average daily gain (ADG), average daily feed intake (ADFI), and gain:feed (G:F) were calculated for each phase. On d 10, 20, and 38 after weaning, a blood sample was obtained from 12 pigs for the isolation of peripheral blood mononuclear cells for monocyte/macrophage assays. After the blood sample was obtained, pigs were rendered unconscious and insensitive to pain by captive bolt stunning and exsanguinated. Gastrointestinal tissue samples were collected to determine intestinal microbiota populations, and to evaluate intestinal morphology and histology.

Sample Processing and Bacterial Enumerations: Gastrointestinal tracts were obtained from the euthanized pigs for coliform and *Escherichia coli* enumeration of the pars esophagous, duodenum, jejunum, and ileum sections. The pars esophageal and duodenum were left attached to the stomach and ligated at the esophageal and duodenum ends and the jejunum and ileum were obtained as separate sections and ligated at their respective ends. All sections were immediately placed in separate whirl-pak bags containing enough sterile saline to cover the entire section of the tract. Tracts were shipped overnight to Agtech Products, Inc. (Waukesha, WI) for processing.

Upon arrival the pars esophageal and duodenum were removed from the stomach. The duodenum was removed from the stomach at the pylorus and a 10 to 15 cm section was obtained. The pars esophageal region was removed at the stomach junction and a 2 to 3 cm section was obtained. The jejunum and ileum sections were obtained at euthanasia as two separate sections, and upon arrival only the ligated ends were removed. All sections were rinsed with sterile phosphate buffer (0.3 mM KH_2PO_4, 1 mM $MgSO_4$, 0.05% cysteine hydrochloride, pH = 7.0) until all contents were washed out and the liquid ran clear. The sections were cut lengthwise to expose the epithelial lining. Each section was weighed in a sterile whirl-pak bag. Sterile phosphate buffer (99 ml) was added to each bag and the contents masticated for 60 seconds. All

samples were plated on CHROMagar (CHROMagar Paris, France) for the enumeration of *E. coli* and Violet Red Bile Agar (Becton, Dickinson, and Company Sparks, Maryland) for the enumeration of coliforms. Spiral plating techniques were used at 10^{-2}, 10^{-4}, and 10^{-5} dilutions on the Autoplate 4000 (Spiral Biotech, Inc., Norwalk, MA). Tissue and bacterial cells were harvested by centrifugation at 5ºC at 10,400 × g for 10 minutes. The supernatant was discarded and the pellet was resuspended in 10 mL MRS broth + 10% glycerol. Samples were stored at -20ºC until further analysis. CHROMagar plates were incubated at 37°C for 24 hours and Violet Red Bile Agar plates were incubated at 37°C for 48 hours before counting on the Q-Count system (Spiral Biotech). The counts were recorded and the section weight and dilution factor was used to obtain the final adjusted *E. coli* and coliform bacterial counts in cfu/g.

Genomic DNA Isolation: The harvested tissue and bacterial cells from each GIT sample were thawed and filtered through a sterile milk filter to remove any large particles. The duodenum, jejunum, and ileal samples were purified further by using 500 µl of each GIT sample in three wash/centrifuge cycles using 10 ml of 10 mM Tris-HCl (pH 7.5) and spins of 1200 × g for 10 minutes. Supernatant was discarded and the pellet was resuspended in 200 µL of 10 mM Tris-HCl pH 8.0. For the pars esophageal samples further purification was not required, instead 500 µL of sample was centrifuged at 1200 × g for 10 minutes. Supernatant was discarded and the pellet was resuspended in 200 µL of 10 mM Tris-HCl pH 8.0. DNA was then isolated using the Roche Applied Science High Pure PCR Template Preparation Kit per the manufacturer's instructions (Mannheim, Germany) except for the following changes: 100 mg/mL of lysozyme was used and incubation was extended to 30 minutes at 37°C, two washes were performed with the inhibitor buffer, and four washes were performed with the wash buffer. The presence of chromosomal DNA product was confirmed on a 0.7% agarose gel stained in 100 µL of 5 µg/mL ethidium bromide per 1000 mL ddH_2O.

PCR: The universal forward primer HDA1 +G/C clamp (**CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGG**ACTCCTACGGG AGGCAGCAGT-3') and reverse primer HDA2 (GTATTACCGCGGCTGCTGGCAC-3') were used to target the V2-V3 region of the 16S rDNA, corresponding to positions 339 to 539 in the *Escherichia coli* gene, described by Muyzer et al. (1993). A 50 µL reaction mixture was used, and consisted of 10X PCR buffer (final concentration 1X), 0.2 mM concentration of each deoxynucleoside triphosphate, 50 mM $MgCl_2$ (final concentration 1.5 mM), 25 pmole of each primer, 30 ng of template DNA, 2.5 units of Platinium Taq Polymerase (Invitrogen, Carlsbad, CA), and sterile water to bring the volume to 50 µL. PCR amplification was carried out in a GeneAmp PCR System 2700 thermocycler (Applied Biosystems, Foster City, CA). The amplification program was 95ºC for 4 min., and 32 cycles of 94ºC for 30 s, 56ºC for 30 s, and 68º C for 1 min., followed by 68ºC for 7 minutes. PCR products were confirmed by a 1% agarose gel stained in 100 µL of 5 µg/mL ethidium bromide per 1000 mL ddH_2O.

Denaturing Gradient Gel Electrophoresis: Parallel 16 cm by 16 cm by 1 mm DGGE gels were cast using the Dcode univeral mutation detection system (BioRad, Hercules, CA) per the manufacturer's protocol. Eight percent polyacrylamide gels were cast and run using 0.5X TAE buffer diluted from 50X TAE buffer (2 M Tris base, 1 M glacial acetic acid, and 50 mM EDTA). The polyacrylamide was diluted from a nondeionized 40% acrylamide/bis stock solution 37.5:1 (Bio-Rad). The gels contained linear gradients of 25-32% urea and formamide increasing in the direction of electrophoresis (100% denaturant *corresponds* to 7 M urea and 40% deionized

formamide). PCR products were applied to gels in aliquots of 20 µL per lane (10 µL of PCR sample + 10 µL of loading dye). Electrophoresis was conducted at 130 V at 60º C for 4 hours. Gels were stained in 0.5X TAE buffer containing 5 µg/mL of ethidium bromide for 10 minutes, and destained in 0.5X TAE buffer for 10 minutes. The gels were then viewed under UV transillumination. Photographs were taken using the Syngene BioImaging system (Syngene, Cambridge, UK). The Bionumerics software program (Applied Maths, Belgium) was used to analyze the DGGE gels.

Intestinal Morphology: Following exsanguination, duodenum (15 cm proximal the pyloric junction), jejunum (55 cm proximal the pyloric junction), and ileum (15 cm distal the ileocaecal junction) were dissected from each pig and placed on ice. Duodenum, jejunum, and ileum samples were cut longitudinally at the antimesenteric attachment and immediately fixed in 10% phosphate-buffered formalin. After fixation, samples were embedded in paraffin. Four to 6 µm sections were cut, mounted on poly-L-lysine-coated slides, and stained with alcian blue (pH 2.5) and periodic acid Schiff (**AB/PAS**) for determination of villus height and crypt depth, and acidic and neutral goblet cells. Sections were also stained with high iron diamine and alcian blue (pH 1.0) for determination of sulfuric acid-containing goblet cells. Positively stained goblet cells were counted on 10 randomly selected villi from each sample. The reported mean value for each pig was based on the 10 measurements. Villus height, area and crypt depth, were evaluated using the Image Pro analysis program (Media Cybernetics, Houston, TX), and were determined by the method of Jaeger et al. (1990) and Nunez et al. (1996).

Immunoglobulin Analysis: After exsanguination, bile was collected for quantification of immunoglobulin-A (IgA) and immunoglobulin-M (IgM). Bile concentrations of IgA were determined using a pig IgA ELISA kit as per the instructions of the manufacturer (Bethyl Laboratories, Montgomery, TX). The dynamic range of the assay was 7.81 to 1,000 pg/mL with a sensitivity of 2.0 pg/ml. The intraassay coefficient of variation for the IgA bile assay was 1.9%. Bile concentrations of IgM were determined using a pig IgM ELISA kit as per the instructions of the manufacturer (Bethyl Laboratories). The dynamic range of the assay was 7.81 to 1,000 pg/mL with a sensitivity of 2.0 pg/ml. The intraassay coefficient of variation was 4.9% for the assay determining the concentration of IgM in bile.

Macrophage Phagocytosis Assay: The method used to measure monocyte/macrophage phagocytic ability of peripheral blood monocytes was adapted from the method of Nibbering et al. (1987) and Heggen et al. (1998). Peripheral blood mononuclear cells were isolated by density gradient centrifugation for the isolation of monocyte/macrophages via adherence to glass surfaces, and to measure their ability to phagocytose sheep red blood cells (SRBC). The incubation temperature for the monocyte/macrophage assay was set at 39.2°C (rather than 37°C) to facilitate monocyte/macrophage activation under conditions simulating pig body temperature (Natale and McCullough, 1998). Briefly, cell suspensions isolated from blood were diluted to approximately 5×10^6 cells/mL in LM Hahn (Leibovitz's L-15/McCoy's Hahn media, Atlanta Biologicals) medium. A glass coverslip was added to each well of a 6-well plate and 2 mL of cell suspension containing monocytes/macrophages was added to each well in duplicate for each sample. Each coverslip was completely covered by cell suspension. Cells were incubated for 5 h at 39.2°C and 5% $C0_2$. Following the 5-h incubation, medium from each well was removed and replaced by 2 mL of fresh LM Hahn medium warmed to 39.2°C. Plates containing cell suspensions were incubated for

an additional 19 h. Following the incubation, plates were removed from the incubator, excess medium was removed from each well, and 2 mL of a 4% sheep red blood cell (SRBC) suspension was added to each well. Plates were incubated with SRBC for 2 h, after which coverslips were removed and non-adherent cells and excess SRBC were washed from the coverslip by rinsing with warmed LM Hahn medium. Coverslips were then stained in Heme 3® (Fischer Scientific, Pittsburg, PA) and percentage of phagocytic monocytes/macrophages and the average number of SRBC consumed by each phagocytic monocyte/macrophage was determined.

Immunohistochemistry: Following exsanguination, samples from the proximal (55 cm away from pyloric junction) small intestine were obtained immediately and snap frozen in liquid nitrogen after being embedded in OCT compound (Tissue Tek II) and stored at –70^{o}C for later analysis. Immunohistochemistry procedures were adapted from the methods of Vega-Lopez et al. (1993). Four to 6 μm frozen sections were obtained, mounted on poly-L-lysine slides, fixed in cold acetone for 15 minutes, and placed in 1 M phosphate buffered saline (PBS) overnight. Sections were then placed in a methanol bath consisting of 0.05% hydrogen peroxide for 20 minutes to quench endogenous peroxidase activity. Sections were then washed with PBS and placed in PBS containing 2.5% heat inactivated horse serum (Atlanta Biologicals, Lawrenceville, Georgia) for 30 minutes at room temperature in a humid chamber. Six commercially available cell molecule-specific, mouse monoclonal antibodies were used to identify pig lymphocyte populations, including T helper lymphocytes (CD4, Southern Biotechnology Associates, Inc., Birmingham, Alabama), cytotoxic T lymphocytes (CD8, Southern Biotechnology Associates, Inc.), activated T and B lymphocytes (CD25 (interleukin-2 receptor), VMRD, Inc., Pullman, WA), and class 2 major histocompatability complex (MHC-II; VMRD, Inc.). Control monoclonal antibodies (Sigma) with irrelevant binding were included to account for non-specific labeling. A 75 μl volume of predetermined optimal dilution of monoclonal antibody was used on the tissue and incubated for 2 h at room temperature in a humid chamber. Affinity purified, biotinylated, horse anti-mouse immunoglobulin (Vector Laboratories, Burlingame, CA) was used as the secondary antibody at a dilution of 1:100 and was incubated for 1 h at room temperature in a humid chamber. Subsequently, the tissues were incubated with a complex of streptavidin-horse radish peroxidase-conjugated biotin (Vector Laboratories). The reaction was visualized using a 0.05 % solution of diaminobenzidine in 1 M PBS (pH 7.6). Between steps, the slides were thoroughly washed in PBS. After visualization, slides were then counterstained in Harris haematoxylin (Sigma), placed through a dehydration bath, mounted with aquamount, and examined under a compound light microscope. Positively stained cells were counted within 10 randomly selected villi per section.

Isolation of Jejunal Intraepithelial Lymphocytes: The isolation procedure for intraepithelial lymphocytes was adapted from the methods of Kearsey and Stadnyk (1996), Poussier and Julius, (1997), Todd et al. (1999) and Solano-Aguilar et al. (2000). Briefly, a 15 cm jejunal sample was incubated on ice for approximately 90 min. Following the cold incubation, samples were flushed with 10 mL of HEPES (N- 2-Hydroxyethylpiperazine-N-2-Ethanesulfuric Acid; Sigma, St. Louis, Missouri) buffered, Hank's balanced salt solution containing 1 mM DTE (Dithioerythritol; Sigma) and 10% fetal bovine serum (Atlanta Biologicals, Atlanta, GA). Weakly adherent epithelial cells and cells within the mucus coating were obtained by gently squeezing the tissue by manual compression. The flushing procedure was repeated five times, and the lumenal eluate containing the intraepithelial lymphocytes was collected in a polystyrene tube.

Cells within the luminal eluate were washed by centrifuging at 350 × g for 5 min, and the resulting pellet was subjected to density gradient centrifugation for further purification by resuspension in 25 mL of a 30% Percoll (Sigma) solution. Cells resuspended in 30% Percoll were centrifuged at 350 × g for 15 min. The supernatant was discarded and the pellet was resuspended in 15 mL of 45% Percoll, underlayed with 15 mL of 75% Percoll, and centrifuged at 350 × g for 30 minutes. Intraepithelial lymphocytes were collected at the interface between the 45% and 75% Percoll layers and washed by centrifuging in RPMI medium at 130 × g for 10 min. Evaluation of isolated cells using a hemocytometer and Trypan Blue (Sigma) exclusion determined leukocyte yield to contain >90% viable cells.

Flow Cytometry Methods: Peripheral blood mononuclear cells and jejunal intraepithelial lymphocytes were isolated as described previously. Cells were resuspended in to minimum concentration of 10^5 cells/mL in RPMI 1640 medium devoid of phenol red (Sigma) for subsequent flow cytometry analysis.

Monoclonal antibodies. A panel of six commercially available mouse monoclonal antibodies were used to identify pig T lymphocytes (CD3, Southern Biotechnology Associates, Inc., Birmingham, Alabama), T helper lymphocytes (CD4, Southern Biotechnology Associates, Inc.), cytotoxic T lymphocytes (CD8, Southern Biotechnology Associates, Inc.), gamma/delta T cell receptor (γ/δ TCR1, VMRD, Inc.), B lymphocytes (CD21, Southern Biotechnology Associates, Inc.), activated T and B lymphocytes (CD25 (interleukin-2 receptor), VMRD, Inc.), and class 2 major histocompatability complex (MHC-II, VMRD, Inc.). Monoclonal antibodies specific for CD3 and CD4 were directly labeled with fluorescein 5(6)-isothiocyanate (FITC), whereas CD21 was directly labeled with R-phycoerythrin (PE). Biotinylated primary antibodies (CD8) were detected by Quantum Red®-labeled streptavidin (Sigma). Primary antibodies specific for MHC-II, γ/δ TCR1, and CD25 were identified with PE- conjugated goat polyclonal antibodies specific for mouse subclasses (Sigma), except in the case of CD21/CD25 double staining procedure in which the CD25 primary antibody was identified with FITC-conjugated goat polyclonal antibodies. Isotype control monoclonal antibodies with irrelevant binding were included to account for non-specific labeling.

Flow cytometry analysis. Control procedures were conducted within each tissue type to test for appropriate binding of monoclonal antibodies to leukocytes of interest. Unlabeled cells were used as a negative control for innate fluorescence detectable in cell suspensions. Isotype controls were used to assess non-specific binding of the directly conjugated monoclonal antibodies, while non-specific binding by labeled secondary antibodies was assessed by incubating cells with unlabeled isotype control in the place of the primary antibody. To conduct compensation (e.g., subtract detection of FL-1 (FITC) from the FL-2 detector (PE), etc.), single color-labeled cell suspensions were used.

Cell suspensions from peripheral blood and jejunal intraepithelium were administered in 50 μL aliquots to wells of a 96-well microtiter plate. Mouse monoclonal antibodies specific for swine cell surface markers were diluted in PBS containing 1% BSA and 0.1% sodium azide (PBS+), and then administered in 50 μL aliquots to appropriate wells at optimal dilutions determined for each antibody during pre-test trials. Plates were then incubated for 30 minutes at 4°C. Following the cold incubation, excess antibody was removed by washing plates twice with 180 μL PBS+ and centrifuging at 180 × g for 4 minutes at 4°C. After washing, if the primary antibody was unconjugated or biotinylated, 50 μL of conjugated goat polyclonal antibodies for mouse immunoglobulin subclasses with an appropriate fluorochrome or Quantum Red®-labeled strepavidin

(Sigma) was administered for 20 min at room temperature for double indirect staining. Excess antibody was removed by washing plates twice with 180 μL PBS+ and centrifuging at 180 × g for 4 minutes at 4°C. Contents of wells were removed and placed into Falcon tubes (Sigma) to evaluate cell populations. Dual fluorescence staining was performed to identify $CD3^+CD8^-$, $CD3^-CD8^+$, $CD3^+CD8^+$, $CD3^-CD8^-$, $CD3^+MHCII^-$, $CD3^-MHCII^+$, $CD3^+MHCII^+$, $CD3^-MHCII^-$, $CD4^+CD8^-$, $CD4^-CD8^+$, $CD4^+CD8^+$, $CD4^-CD8^-$, $CD21^+CD25^-$, $CD21^-CD25^+$, $CD21^+CD25^+$, and $CD21^-CD25^-$ lymphocyte proportions.

For triple indirect staining, cells double-stained for CD4 and CD8 were incubated for 30 min at 4°C with 50 μl of unconjugated monoclonal antibodies specific for CD25 (IL-2) or γ/δ TCR. Following the cold incubation, excess antibody was removed by washing plates twice with 180 μL PBS+ and centrifuging at 180 × g for 4 minutes at 4°C. After washing, 50 μL of PE-conjugated goat polyclonal antibody for mouse immunoglobulin subclasses was administered for 20 min at room temperature. Excess antibody was removed by washing plates twice with 180 μL PBS+ and centrifuging at 180 × g for 4 minutes at 4°C. Contents of wells were removed and placed into Falcon tubes (Sigma) to evaluate cell populations.

A FACSort flow cytometer and CellQuest™ software (Becton-Dickinson Immunocytometry Systems, San Jose, California) was used to conduct one-, two- and three-color analysis of stained cell populations.

Statistical Analysis: Data were analyzed as a completely randomized design during the lactation phase and as a 2 x 3 factorial in a randomized complete block design with pen as the experimental unit during the nursery phase. Least square means were compared with F-protected ($P < .05$) t-test. Analyses of variance were performed using the GLM procedure of SAS (SAS Inst. Inc., Cary, NC).

Results:

Performance Measures: Because no significant interactions ($P > 0.15$) were observed between the administration of 1E1 and dietary nursery treatments, main effect means are presented. The effect of 1E1 on growth performance and pig survival during lactation and growth performance during the nursery phase of the study are presented in Tables 4 and 5, respectively. Inclusion of 1E1 in the milk supplement during lactation had no significant effect ($P > 0.30$) on average daily gain (ADG), body weight (BW) or survival of pigs to weaning when compared to pigs fed milk supplement without 1E1 (Table 4). During the nursery period, pigs administered 1E1 had greater ADG ($P < 0.01$) during Phase 2 and in the overall nursery period (Table 5, $P < 0.05$). Increased gain of pigs supplemented with 1E1 resulted in a 0.8 and 1.5 kg improvement ($P < 0.01$) in body weight at the end of Phase 2 and Phase 3, respectively, when compared to those not receiving 1E1. Similarly, ADFI was improved during Phase 3 and in the overall nursery period in pigs receiving 1E1 when compared to those not receiving 1E1 ($P < 0.05$). Feed efficiency (gain:feed) tended to decrease ($P < 0.10$) during Phase 3 in pigs administered 1E1 when compared to pigs not supplemented with 1E1.

Main effect means for the dietary nursery treatments are presented in Table 6. Pigs fed antibiotics had greater ADG ($P < 0.01$) during Phase 2 and during the overall nursery period when compared to pigs fed the negative control diet or those fed *Bacillus* cultures. Improvement in gain of pigs fed antibiotics resulted in an approximately 2 kg increase in body weight ($P < 0.01$) at the end of Phase 2 and the overall nursery period when compared to pigs fed the control diet or *Bacillus* cultures. Average daily feed intake was greater ($P < 0.01$) in pigs receiving antibiotics during Phase 3 and in the overall nursery period when compared to those fed the control diet or *Bacillus* cultures.

Average daily gain and ADFI was similar among pigs fed *Bacillus* cultures and those fed the control diet. During Phase 2, pigs supplemented with antibiotics had improved (P < .05) G:F when compared to pigs fed *Bacillus* cultures; however, during Phase 3 pigs supplemented with *Bacillus* cultures had improved (P < 0.01) G:F when compared to pigs fed the control diet or antibiotics.

Escherichia coli and Coliform Enumeration: Differences in the quantification of *E. coli* and coliform populations on each of the sampling days were observed primarily in the duodenum and the jejunum of the piglet small intestine (Table 7). The adjusted counts (log 10) of *E. coli* and coliforms were greatest (P < 0.05) in both tissue sections on d 20 after weaning compared to d 38 after weaning. Numerically, adjusted *E. coli* and coliform counts were greater in the ileum than in the duodenum and jejunum, however their quantity remained consistent in the ileum over all three sampling days.

Treatments administered in this study affected *E. coli* and coliform counts in the duodenum and jejunum mostly on the days when the overall quantities of the measured microbes were greatest. In the duodenum, pigs administered 1E1 had greater (P < 0.05) coliform counts on d 10 after weaning compared to pigs that did not receive 1E1, however, coliform counts were highest on d 20 after weaning in pigs that did not receive 1E1 and tended to be greater (P = 0.10) than coliform counts from pigs administered 1E1 (1E1 x day interaction, P < 0.05; Figure 1). In the jejunum, the greatest adjusted *E. coli* counts were observed on d 20 after weaning in pigs not provided with 1E1, and these counts were significantly greater (P < 0.05) than those observed on d 20 after weaning when pigs were administered 1E1. The administration of 1E1 did not affect adjusted *E. coli* counts within the jejunum on d 10 or d 38 after weaning (1E1 x day interaction, P < 0.05; Figure 2). Also, the overall response of adjusted *E. coli* counts within the jejunum to the three dietary treatments during the nursery period was dependent upon 1E1 administration (1E1 x nursery diet interaction, P = 0.05; Figure 3). Numerically, adjusted *E. coli* counts were less when the control and antibiotic diets were fed with 1E1 administration compared to providing the control and antibiotic diets without 1E1; however, adjusted E. coli counts tended to be higher (P < 0.06) in pigs fed *Bacillus* with 1E1 compared to pigs fed *Bacillus* without 1E1.

DGGE Analysis: Banding patterns of 16S rDNA from the intestinal microflora of pig intestinal samples were compared on denaturing gradient gels using Bionumerics software (Applied Maths, Belgium). Recognition of gel banding patterns and discriminative analysis were performed to distinguish those bands correlated with 1E1 administration or dietary treatment. Differences were observed in the microbial community profiles generated by DGGE for each different intestinal segment. However, some trends were observed with regard to 1E1 and dietary treatments. For example, the microbial profiles of jejunal sections of pigs administered 1E1 contained an intense band of low G/C content that was not present in many of the jejunal samples from pigs not administered 1E1 (Figure 4A). Likewise, in the jejunal samples from pigs not administered 1E1 a faint, moderate G/C content band was present that was typically absent from jejunal samples of animals given 1E1 (Figure 4B).

Intestinal Morphological Measurements: The administration of 1E1 did not affect duodenal villus height (264.2 vs. 255.7 μm for pigs not administered 1E1 and pigs provided 1E1, respectively) or duodenal crypt depth (88.2 vs. 86.7 μm for pigs not administered 1E1 and pigs provided 1E1, respectively) in the overall nursery period. However, pigs provided 1E1 had a lower (P < 0.05) villus height:crypt depth ratio in the duodenum on d 38 after weaning compared to pigs not administered 1E1, whereas

there was no difference in the villus:crypt ratio between the 1E1 treatments on d 10 and d 20 after weaning (1E1 x day interaction, $P < 0.01$; Figure 5). Moreover, a similar response to 1E1 treatment was observed for the villus height:crypt depth ratio in the jejunum of pigs (1E1 x day interaction, $P < 0.01$; Figure 6). In the ileum, villus height was greater ($P < 0.05$) on d 10 after weaning when pigs were supplemented with 1E1 compared to pigs not provided 1E1, although villus height was greater ($P < 0.05$) for pigs not provided 1E1 on d 38 after weaning (1E1 x day interaction, $P < 0.01$; Figure 7). Villus area within the ileum was also greater ($P < 0.05$) for pigs not provided with 1E1 compared to pigs administered 1E1 on d 38 after weaning, but did not differ in response to 1E1 treatment on d 10 or d 20 after weaning (1E1 x day interaction, $P < 0.01$; Figure 8). In the ileum, villus height was greater ($P < 0.05$) in pigs fed the antibiotic diet compared to pigs fed the control diet or *Bacillus* on d 10 after weaning, however, pigs fed *Bacillus* exhibited greater ($P < 0.05$) villus height within the ileum on d 38 after weaning compared to pigs fed the control diet (nursery diet x day interaction, $P < 0.01$; Figure 9).

In the duodenum, the administration of 1E1 decreased ($P < 0.05$) the number of sulfated mucin-producing goblet cells while increasing ($P < 0.05$) the number of acidic sialylated goblet cells, compared to pigs that did not receive 1E1 (Table 8). Pigs fed diets containing *Bacillus* and antibiotic had a lower ($P < 0.05$) number of sulfated mucin-producing goblet cells in the duodenum than pigs fed the control diet during the nursery period. Also, acidic sialylated were more abundant in the jejunum of pigs administered 1E1 compared to pigs not provided 1E1, whereas sulfated mucin-producing goblet cells were less abundant in the jejunum when pigs were provided with 1E1 (Table 8). The number of sulfated mucin-producing goblet cells was greater ($P < 0.05$) on d 10 and on d 20 after weaning in pigs fed the control diet compared to pigs fed *Bacillus* and pigs fed the antibiotic diet, whereas there was no difference in the number of sulfated mucin-producing goblet cells among the three dietary treatments on d 38 after weaning (nursery diet x day interaction, $P < 0.01$; Figure 10). In addition, on d 20 after weaning, the number of sulfated mucin-producing goblet cells was lowest ($P < 0.05$) when pigs were fed *Bacillus* compared to pigs fed the control or antibiotic diets (Figure 10). In the ileum, the number of acidic sialylated goblet cells was greater ($P < 0.05$) on d 20 and d 38 after weaning when pigs were administered 1E1 compared to pigs not provided with 1E1, whereas there was no difference in the number of acidic goblet cells in the ileum on d 10 after weaning, regardless of 1E1 supplementation (1E1 x day interaction, $P < 0.01$; Figure 11).

Monocyte/Macrophage Phagocytosis: Pigs fed the control diet and those fed *Bacillus* had a greater ($P < 0.09$) percentage of phagocytic monocyte/macrophages than pigs fed the antibiotic diet in the absence of 1E1 administration. However, with 1E1 administration, pigs fed *Bacillus* had fewer phagocytic monocyte/macrophages than pigs fed the control diet; whereas the response of pigs fed the antibiotic diet with 1E1 was intermediate between those pigs fed the control diet and those fed *Bacillus* (1E1 x diet interaction, $P < 0.10$; Figure 12). Regardless of 1E1 administration, monocyte/macrophages isolated from pigs fed *Bacillus* consumed a greater ($P < 0.05$) number of sheep red blood cells than pigs fed the antibiotic diet, whereas monocyte/macrophages isolated from pigs fed the control diet were intermediate between the *Bacillus* and antibiotic treatments (Table 9).

Jejunal Immune Cell Populations (as determined by Immunohistochemistry and flow cytometric analysis of intraepithelial lymphocytes): No differences in immune cell populations within the jejunum of the gastrointestinal tract were detected among the

treatments administered in this experiment by immunohistological analysis (Table 10). Additionally, the isolation procedure to yield intraepithelial lymphocytes failed to produce cells in adequate quantity for flow cytometric analysis. Therefore, intraepithelial lymphocyte proportions from flow cytometric analysis are not reported.

Peripheral Blood Mononuclear Cell Populations (as determined by flow cytometric analysis): The proportion of T cells (as indicated by the presence of the CD3 cell surface marker) within peripheral blood mononuclear cells did not differ ($P > 0.05$) regardless of 1E1 administration or dietary supplementation on d 10 after weaning, however experimental treatments altered T cell proportions on d 20 and 38 after weaning (1E1 x diet x day interaction, $P = 0.03$; Table 11 and Figure 13). On d 20 after weaning, the proportion of $CD3^+$ T cells were higher ($P < 0.05$) in pigs fed the antibiotic diet in the absence of 1E1 and those administered 1E1 and fed the control diet compared to pigs fed the control and *Bacillus* diets in the absence of 1E1 and pigs administered 1E1 and fed *Bacillus*. However, on d 38 after weaning, pigs fed *Bacillus* diets both in the presence and absence of 1E1 administration had a higher ($P < 0.05$) proportion of $CD3^+$ T cells than pigs in any of the other experimental treatments. This same pattern was evident when evaluating the proportion of $CD3^+$ T cells expressing the MHCII molecule ($CD3^+MHCII^+$ cells, 1E1 x diet x day interaction, $P = 0.04$; Table 12 and Figure 14). Although there was no difference ($P > 0.10$) in the proportion of peripheral blood mononuclear cells expressing the MHCII molecule among the six experimental treatments on d 10 or d 38 after weaning, a higher ($P < 0.05$) proportion of peripheral blood mononuclear cells isolated from pigs fed the *Bacillus* or antibiotic diets in the presence or absence of 1E1 and those fed the control diet in the presence of 1E1 was observed on d 20 after weaning compared to cells isolated from pigs fed the control diet in the absence of 1E1 (1E1 x diet x day interaction, $P = 0.04$; Table 11 and Figure 15). A nursery diet x day interaction ($P < 0.01$) was observed for $CD3^-MHCII^+$ (representing the MHCII-expressing monocyte and B cell populations) leukocytes within peripheral blood mononuclear cells, such that there were no differences ($P > 0.05$) in the proportion of $CD3^-MHCII^+$ cells on d 10 or d 38 after weaning in response to dietary treatments, however on d 20 after weaning, pigs fed the *Bacillus* and antibiotic diets had a higher ($P < 0.05$) proportion of $CD3^-MHCII^+$ cells than pigs fed the control diet (Table 12).

The proportion of peripheral blood mononuclear cells expressing the CD4 T lymphocyte molecule (indicative of T helper cells) did not differ in response to dietary treatments on d 10 after weaning, however pigs fed diets containing *Bacillus* or antibiotic had a higher proportion of $CD4^+$ lymphocytes compared to pigs fed the control diet on d 20 after weaning, whereas pigs fed the control and *Bacillus* diets had higher proportions of $CD4^+$ lymphocytes compared to pigs fed the antibiotic diet on d 38 after weaning (nursery diet x day interaction, $P = 0.02$; Table 11). Pigs fed *Bacillus* and administered 1E1 had a higher ($P < 0.05$) proportion of $CD8^+$ T cells within peripheral blood mononuclear cells than pigs fed the control diet in the absence of 1E1 on d 10 after weaning. However, on d 20 after weaning, pigs fed *Bacillus* and antibiotic in the absence of 1E1 and pigs fed all three diets and administered 1E1 had a higher ($P < 0.05$) proportion of $CD8^+$ T cells compared to pigs fed the control diet without 1E1 administration, whereas on d 38 after weaning, the proportion of $CD8^+$ T lymphocytes was higher ($P < 0.05$) in pigs fed all three diets in the absence of 1E1 and those fed antibiotics in the presence of 1E1compared to pigs fed the control and *Bacillus* diets in the presence of 1E1 (1E1 x diet x day interaction, $P = 0.001$; Table 11 and Figure 16). The proportion of $CD4^-CD8^+$ peripheral blood mononuclear cells increased ($P < 0.05$) from d 10 to d 38 after weaning; and while the administration of 1E1 did not influence (P

> 0.05) the proportion of $CD4^-CD8^+$ T lymphocytes within the peripheral blood on d 10 and d 38 after weaning, 1E1 administration increased (P < 0.05) the proportion of these cells on d 20 after weaning (1E1 x day interaction, P < 0.01, Table 13).

The proportion of $\gamma\delta$ T cells (as indicated by the presence of TCR1) within the peripheral blood mononuclear cell population did not differ (P > 0.05) in response to the six experimental treatments on d 10 or d 38 after weaning; whereas on d 20 after weaning, pigs fed the antibiotic diet in the absence of 1E1 and those fed the control diet with 1E1 administration had a higher (P < 0.05) proportion of $\gamma\delta$ T cells than pigs fed the control and *Bacillus* diets in the absence of 1E1 and those fed *Bacillus* in the presence of 1E1. In addition, the proportion of $\gamma\delta$ T cells was higher (P < 0.05) in pigs fed *Bacillus* both in the presence and absence of 1E1 compared to pigs fed the control diet without 1E1 administration (1E1 x diet x day interaction, P = 0.04; Table 11 and Figure 17).

The proportion of peripheral blood mononuclear cells expressing CD25 (indicative of T cell activation by exposure to antigen) was not altered (P > 0.05) by dietary treatments on d 10 after weaning. However pigs fed the *Bacillus* or antibiotic diets had higher (P < 0.05) proportions of CD25 compared to pigs fed the control diet on d 20 after weaning, whereas on d 38 after weaning, pigs fed the control and *Bacillus* diets had a higher (P < 0.05) proportion of these cells compared to pigs fed the antibiotic diet (nursery diet x day interaction, P = 0.07; Table 11). On d 10 after weaning, pigs fed the *Bacillus* diet in the absence of 1E1 had a higher (P < 0.05) proportion of $CD4^+CD8^-$ lymphocytes within the $CD25^+$ population ($CD25^+CD4^+CD^-$) than pigs fed the control diet without 1E1 (Figure 18). Although experimental treatments did not alter (P > 0.05) this cell population on d 20 after weaning, the proportion of $CD4^+CD8^-$ within the $CD25^+$ population was higher (P < 0.05) in pigs fed *Bacillus* in the presence of 1E1 compared to pigs fed *Bacillus* and antibiotic in the absence of 1E1 and the control and antibiotic diets in the presence of 1E1 (1E1 x diet x day interaction, P = 0.04; Figure 18). Although the proportion of $CD25^+$ lymphocytes within the $CD4^+CD8^+$ T cell population ($CD25^+CD4^+CD8^+$) did not differ (P > 0.05) on d 20 and d 38 after weaning, on d 10 after weaning, the proportion of this population was higher (P < 0.05) in pigs fed the control and *Bacillus* diets in the presence of 1E1 compared to pigs fed the same diets in the absence of 1E1 and those fed the antibiotic diet in the presence of 1E1 (1E1 x diet x day interaction, P = 0.004; Figure 19). In addition, the proportion of $CD25^+$ lymphocytes within the $CD4^+CD8^+$ T cell population was higher (P < 0.05) in pigs fed the *Bacillus* diet without 1E1 and the antibiotic diet with 1E1 compared to pigs fed the control diet in the absence of 1E1 (Figure 19).

Discussion: In the present study, the supplementation of 1E1 during the suckling phase and throughout the nursery period improved body weight gain and feed conversion in pigs. These findings are similar to observations made by Davis et al. (2003), in which ADG and G:F were greater during the post-weaning period in pigs receiving 1E1 compared to control pigs not provided with 1E1. The similar effects from the two studies indicate a consistent effect of 1E1 on growth and performance of nursery pigs. These beneficial effects may result from the improvement of intestinal health and the growth inhibition of pathogenic bacteria. It has been reported that piglets at birth are devoid of bacteria in the intestine (Abe et al, 1995). Therefore, the administration of 1E1 during the pre-weaning phase may promote early colonization in the intestine by beneficial bacteria that may act as competitive excluders of pathogenic bacteria.

The use of antibiotics in livestock feed has been well documented (Cromwell, 2001), and as expected, antibiotic supplementation increased ADG and ADFI during the

post-weaning period in the present study, compared to pigs fed *Bacillus* and the control diet. Supplementation of *Bacillus* cultures has been reported to improve growth performance in weanling pigs (Yang et al., 2003; Adami et al., 1997), however, results have been variable. In this study, performance of pigs was not affected by *Bacillus* culture administration until Phase 3 of the post-weaning period, in which G:F was greater in pigs fed *Bacillus* than pigs fed the antibiotic or the control diet. Although this effect was not evident in the evaluation of the overall nursery period, we surmised that this particular *Bacillus* culture may be effective to the host animal but the effect is gradual. Unlike 1E1, *Bacillus* cultures were only administered during the post-weaning stage and may need some time for the impact to become evident. In addition, the *Bacillus* were selected to have antimicrobial activity against F18 and K88 *E. coli*, and there was no evidence that these isolates of *E. coli* were present in this study.

Although duodenal coliform counts were higher on d 10 after weaning in pigs provided 1E1, both duodenal coliform and jejunal E. coli counts were lower in 1E1-supplemented pigs on d 20 after weaning. Interestingly, d 20 after weaning corresponds with the Phase 2 time period during weaning in which the beneficial response to 1E1 supplementation was observed. Previous experiments with 1E1 have demonstrated that weanling pigs administered 1E1 had lower jejunal *E. coli* populations compared to control pigs (Davis et al., 2003). Studies have shown that the weaning process significantly increases the number of potentially pathogenic coliforms, such as haemolytic *E. coli* (Hampson *et al*., 1985), and reduces favorable lactobacilli (Bolduan, 1999) in the small intestine of the piglet. The results of this study suggest that 1E1 may impact pig growth performance by colonizing sites within the digestive tract and thereby excluding invading pathogens.

Denaturing gradient gel electrophoretic patterns of the 16S rDNA from the microflora of the pig intestinal tracts indicated that there may exist important differences in the gut microbial communities of pigs fed different dietary treatments or supplemented with 1E1. A clear example of this was observed in the banding patterns of jejunal microflora. Here, a prominent band appeared in the patterns of pigs administered 1E1 which was not usually observed in the banding patterns of pigs not given 1E1. Similarly, a different band was present primarily in samples from pigs not given 1E1. Further analysis involving extraction and sequencing of these unique bands would allow comparison of their 16S sequence to those in GenBank's NCBI database. This would allow the obtainment of information regarding the possible species origins of the excised DNA fragments and may provide further insight into the changes occurring in the microbial assemblages of the pig gastrointestinal tract as different dietary treatments are administered. This information could be invaluable in identifying both microorganisms that have a beneficial impact on the health or performance of piglets and microorganisms that have detrimental effects on health and performance.

Gastrointestinal morphological measurements (villus height, crypt depth, and villus area) did not correspond to improvements in pig growth performance in this study. The administration of 1E1 did result in alterations in the villus architecture, however villus:crypt ratios were variable compared to control pigs depending upon time after weaning. Villus height:crypt depth ratios have been reported to be altered in response to different weaning environments and populations of the resident gastrointestinal microflora (Tang et al., 1999). Additionally, a study by Abrams (1977) demonstrated that the resident bacterial flora caused shorter villi and deeper crypts when compared to germ-free animals, suggesting that more functionally immature epithelial cells were present at the villus tip of conventionally managed animals due to a more rapid migration of epithelial cells from the crypts. However, shorter villi and deeper crypts were associated with increased performance in 1E1 pigs in the present study.

Conversely, greater villus height was observed in pigs provided antibiotics during the nursery period, and this increase corresponded to improvements in pig performance observed from antibiotic supplementation.

Although 1E1 did not result in consistent improvement in the gastrointestinal morphological architecture, beneficial changes were observed in the number and type of mucin-producing goblet cells. Interspersed among the absorptive cells of the intestinal epithelia, goblet cells function in the synthesis of water-soluble mucins and trefoil peptides to form a continuous gel on the mucosal surface (Kindon et al., 1995; Matsuo et al., 1997). The mucus layer constitutes a physical barrier between the lumen and epithelium as well as an important framework for host-bacteria and bacteria-bacteria interactions (Bourlioux et al., 2002). Goblet cell numbers as well as the production of mucins from these cells are affected by the weaning process. In a study conducted by Dunsford and coworkers (1991), goblet cell numbers in the villi decreased after the weaning process and began to increase in number by 3 days post-weaning. And, although goblet cells numbers were increased by 3 days after weaning, the populations of neutral and acidic goblet cells were decreased. Another study by Brown and coworkers (1988) observed significant changes in the population of sulfated and acidic goblet cells within the jejunum and ileum after weaning in response to the pigs' rearing environment.

Mucin-containing cells may also be influenced by the effects of the microbial flora on the differentiation of mucin-secreting cells from the crypt epithelial cells (Sharma et al., 1995). These effects may be related to alterations in villus and crypt lengths within the small intestine which can affect the normal differentiation and maturation of the goblet cells. Goblet cells differentiate from the endodermal stem cells deep within the crypts in the small intestine and as they mature, they migrate to the villus tip and are eventually sloughed off (Dunsford et al., 1991). Furthermore, as these cells are migrating from the crypt to the villi the chemical composition of the mucin within the cell is also changing. As detected by histochemical methodology, the immature goblet cells deep within the crypts of the small intestine produce neutral mucins containing little sialic acid and as they mature and migrate to the villus tip, the mucins become increasingly sialated, or acidic (Specian and Oliver, 1991). In the present study, the administration of 1E1 increased the number of acidic goblet cells in the duodenum and in the jejunum compared to pigs not provided with 1E1, suggesting a maturity in the development of mucus-secreting goblet cells.

The effect of the administration of 1E1 on the percentage of phagocytic monocyte/macrophages was dependent upon the dietary treatments administered during the nursery period. An increased percentage of monocyte/macrophages suggests a more inflammatory condition was present in the animals from which these cells were isolated. For instance, phagocytosis is an important defense mechanism when invading pathogens are present, however an increased state of inflammation is also associated with the pro-inflammatory response that results in decreased feed intake and metabolic alterations detrimental to growth (Spurlock, 1997). As observed in the present study, we would expect the inflammatory environment to be reduced when pigs are provided antibiotics due to the reduced pathogen load. Interestingly, a similar decrease in the percentage of phagocytic monocyte/macrophages was observed for pigs administered both 1E1 and *Bacillus* as that observed for pigs provided antibiotic supplementation, suggesting that the combination of these direct-fed microbials promotes the same quiescence of the inflammatory response that results from the administration of antibiotics. However, functionally, the monocyte/macrophages isolated from pigs supplemented with 1E1 and *Bacillus* were not compromised, such that in the presence of a challenge (in this study, simulated by the administration of SRBC in

culture), more SRBC were consumed by monocye/macrophages isolated from 1E1-fed pigs compared to pigs not provided with 1E1 (although not statistically significant), and more were consumed by *Bacillus*-fed pigs than those fed the control or antibiotic diet.

Interestingly, in pigs provided 1E1, *Bacillus*, or antibiotics, the expression of MHC-II on peripheral blood mononuclear cells was increased compared to control pigs, suggesting that the capacity of antigen presenting cells to process and present antigen to $CD4^+$ lymphocytes is promoted by 1E1, *Bacillus*, and antibiotic supplementation. Also, this increased MHC-II expression, indicative of antigen presenting ability, was most evident on d 20 after weaning when coliform and *E. coli* populations were highest, suggesting that this may be an important timepoint in post-weaning immunological development. Further evidence for the importance of the d 20 post-weaning timepoint were the increases in the proportion of peripheral blood mononuclear cells expressing CD8 and the $\gamma\delta$-T cell receptor observed in all treatments compared to pigs fed the control diet in the absence of 1E1. The expression of MHC-II on $CD3^+$ peripheral blood mononuclear cells was greatest in pigs fed antibiotics in the absence of 1E1 supplementation and in pigs fed the control and the Bacillus diet in the presence of 1E1. The presence of MHC-II on human T cells has been confirmed (Hewitt and Feldmann, 1989), although its presence on murine T cells is somewhat controversial and seems to be an artifact of laboratory techniques (Lorber et al., 1982). The expression of MHC-II on T lymphocytes is present only under certain conditions of activation of the T lymphocyte, after recognition of specific antigen complexed to MHC-II of antigen presenting cells by the T cell receptor, and its presence suggests that activated T lymphocytes have the ability to process and present antigen to other T cells (Holling et al., 2002). These data indicate that porcine T cells have the ability to express MHC-II and present antigen when activated, and that the aforementioned treatments resulted in an increase in an activated population of T lymphocytes in the peripheral blood. Whether these MHC-II^+ T cells have a stimulatory function in T cell responses or down-regulate by inducing anergy in activated T cells remains in question (Barnaba et al., 1994; Pichler and Wyss-Coray, 1994).

Immunoregulatory function appears to be primarily governed by a subset of $CD4^+$ T cells expressing the CD25 T cell activation marker (Thompson and Powrie, 2004). These $CD4^+CD25^+$ T cells may be naturally occurring (thymically derived), or they may be induced from naïve $CD4^+$ T cells upon activation by antigen exposure (Piccirillo and Thornton, 2004). These regulatory T cell subsets are defined by the presence of CD4 and CD25 cell surface markers as well as their cytokine profiles, such that $CD4^+CD25^+$ regulatory T cells produce IL-10 and transforming growth factor-β, cytokines that are known to inhibit inflammatory immune reactions and induce tolerance (O'Garra and Vieira, 2004). Although in our present study we did not evaluate cytokine profiles, the increased proportion of $CD4^+CD8^-CD25^+$ T cells in pigs fed *Bacillus* in the absence of 1E1 at d 10 post-weaning and in pigs fed *Bacillus* in the presence of 1E1 at the end of the trial suggests that supplementation with *Bacillus* supports the development of this regulatory T cell subset. Furthermore, this corroborates our interpretation that the observed decrease in the percentage of phagocytic macrophages with 1E1 and *Bacillus* in combination suggests a decrease in the inflammatory environment, as the $CD4^+CD25^+$ T cell subset may potentiate the suppression of inflammatory processes that may prohibit growth and efficiency.

The results of the present study indicate the efficacy of the administration of 1E1 to improve the growth rate of nursery pigs. *Lactobacillus brevis* 1E1 administration also resulted in positive alterations of gastrointestinal morphology after weaning, as indicated by goblet cell enumerations, which suggest the development of a more mature gastrointestinal epithelial structure. Lastly, the combination of 1E1 and *Bacillus* should

be explored further for its propensity to promote the development of the $CD4^+CD25^+$ regulatory T cell subset, and to decrease inflammatory immune responses similar to pigs supplemented with growth-promoting levels of antibiotics.

Lay Interpretation: Direct-fed microbials may hold promise as an alternative to growth promoting additions of antibiotics added to swine diets. A study conducted at the University of Arkansas evaluated the effects of two direct-fed microbials (*Lactobacillus brevis* and a *Bacillus* culture) on growth, gastrointestinal microflora, and immune system characteristics of weanling pigs compared to antibiotic supplementation with Carbodox (generic name?). Both supplementation with *Lactobacillus brevis* and the antibiotic resulted in improved average daily gain, feed intake, and body weight at the end of the trial compared to pigs fed a basal diet. In addition, *Lactobacillus brevis* supplementation altered the gastrointestinal microbial population. Different bacterial populations were present in pigs provided *Lactobacillus brevis* that were absent in pigs not administered 1E1, and similarly, populations were present in pigs not provided *Lactobacillus brevis* that were absent in pigs exposed to *Lactobacillus brevis*. Furthermore, the combination of *Lactobacillus brevis* and *Bacillus* seems to result in beneficial immune responses characteristic of decreased inflammation similar to antibiotic supplementation. For further information, please contact Dr. Charles Maxwell at 479-575-2111 or cmaxwell@uark.edu.

Literature Cited

Abe, F., N. Ishibashi, and S. Shimamura. 1995. Effect of administration of Bifidobacteria and lactic acid bacteria to newborn calves and piglets. J. Dairy Sci. 78:2838-2846.

Abrams, G. D. 1977. Microbial effects on mucosal structure and function. Am. J. Clin. Nutr. 30:1880.

Adami, A., A. Sandrucci and V. Cavazzoni. 1997. Piglets fed from birth with the probiotic *Bacillus* coagulans as additive: zootechnical and microbiological aspects. Ann. Microbiol. Enzimol. 47: 139-149.

Banach, S., T. Rehberger, T. Parrott, C. Maxwell, J. Coalson and K. Touchette. 2002. Influence of *Lactobacillus brevis* 1E-1 on the gastrointestinal microflora of pre-weaning and weaning pigs J. Anim. Sci 80(Suppl. 1):248.

Barnaba, V., C. Watts, M. de Boer, P. Lane, and A. Lanzavecchia. 1994. Professional presentation of antigen by activated human T cells. Eur. J. Immunol. 24:71.

Barrow, P. A., A. R. Fuller, and N. J. Newport. 1977. Changes in the microflora and physiology of the anterior intestinal tract of pigs weaned at 2 days, with special reference to the pathogenesis of diarrhea. Infect. Immun. 18:586-595.

Bolduan, G. 1999. Feeding weaner pigs without in-feed antibiotics. Page 223-230 in Biotechnology in the Feed Industry. Proceedings of Alltech's 15th Annual Symposium. T. P. Lyons and K. A. Jacques, ed. Nottingham University Press, Nottingham, UK.

Bourlioux, P., B. Koletzko, F. Guarner, and V. Braesco. 2002. The intestine and its microflora are partners for the protection of the host: report on the Danone Symposium "The intelligent Intestine". Am. J. Clin. Nutr. 78(4):675.

Brown, P. J., B. G. Miller, C. R. Stokes, N. B. Blazquez, and F. J. Bourne. 1988. Histochemistry of mucins of pig intestinal secretory epithelial cells before and after weaning. J. Comp. Pathol. 98:313.

Cromwell, G. L. 2001. Antimicrobial and promicrobial agents. In: A. J. Lewis and L. L. Southern (eds.) Swine Nutrition. P. 611. CRC Press, Boca Raton, FL.

DANMAP. DANMAP 2002-Use of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from food animals, foods, and humans in Denmark. ISSN 1600-2032.

Davis, M.E., D.C. Brown, Z.B. Johnson, and C.V. Maxwell. 2003. Influence of *Lactobacillus brevis* 1E1 on the gastrointestinal microflora, gut morphology, and growth performance of weanling pigs pre- and post weaning. Arkansas Anim. Sci. Dept. Report. 161.

Dawson, K. A., B. E. Langlois, T. S. Stahly, and G. L. Cromwell. 1984. Antibiotic resistance in anaerobic and coliform bacteria from the intestinal tract of swine fed therapeutic and subtherapeutic concentrations of chlortetracycline. J. Anim. Sci. 58:123-131.

Dunsford, B. R., W. E. Haensly, and D. A. Knabe. 1991. Effects of diet on acidic and neutral goblet cell populations in the small intestine of early weaned pigs. Am. J. Vet. Res. 52(10):1743.

Gaskins, H. R.. 2001. Intestinal bacteria and their influence on swine growth In: Austin J. Lewis and Lee L. Southern (Ed.). Swine Nutrition 2nd Edition. P 585-608.

Hampson, D. J., M. Hinton, and D. E. Kidder. 1985. Coliform numbers in the stomach and small intestine of healthy pigs following weaning at three weeks of age. J. Comp. Pathol. 95(3):353.

Hathaway, M. R., W. R. Dayton, M. E. White, T. L. Henderson, D. A. Young, and T. N. Doan. 1999. Effect of intake on antibiotically induced increases in porcine serum insulin-like growth factor I. J. Anim. Sci. 77:3208.

Heggen, C. L., M. A. Qureshi, F. W. Edens, H. J. Barnes, and G. B. Havenstein. 1998. Alterations in the lymphocytic and mononuclear phagocytic systems of turkey poults associated with exposure to poult enteritis and mortality syndrome. Avian Dis. 42:711-720.

Hewitt, C. R. A., and M. Feldmann. 1989. Human T cell clones present antigen. J. Immunol. 143:762-769.

Jaeger, L., C. Lamar, T. Cline, and C. Cardona. 1990. Effect of orally administered epidermal growth factor on the jejunal mucosa of weaned pigs. Am. J. Vet. Res. 51(3):471.

Kearsey, J. A. and A. W. Stadnyk. 1996. Isolation and characterization of highly purified rat intestinal intraepithelial lymphocytes. J. Immunol. Methods. 194:35-48.

Kindon H, C. Pothoulakis, L. Thim, K. Devaney, and D. K. Podolsky. 1995. Trefoil peptide protection of intestinal epithelial barrier function: cooperative interaction with mucin glycoprotein. Gastroenterology 109:516.

Lorber, M. I., M. R. Loken, A. M. Stall, and F. W. Fitch. 1982. I-A antigens on cloned alloreactive murine T lymphocytes are acquired passively. J. Immunol. 128:2798.

Mackie, R. I., A. Sghir, and H. R. Gaskins. 1999. Developmental microbial ecology of the neonatal gastrointestinal tract. Am. J. Clin. Nutr. 69(Suppl.):1035S-1045S.

Matsuo K, H. Ota, T. Akamatsu, A. Sugiyama, T. Katsuyama. 1997. Histochemistry of the surface mucous gel layer of the human colon. Gut 40:782–9.

Matthew, A. G., W. G. Upchurch, and S. E. Chattin. 1998. Incidence of antibiotic resistance in fecal *Escherichia coli* isolated from commercial swine farms. J. Anim. Sci. 76:429-434.

Muyzer, G., E. C. De Waal, and A. G. Uitterlinden. 1993. Profiling of complex microbial populations by denaturing gradient gel electophoresis analysis of polymerase chain reaction-amplified genes coding for 16s rRNA. Appl. Environ. Microbiol. 59:695-700.

Natale, V. A. I., and K. C. McCullough. 1998. Macrophage culture: influence of species-specific incubation temperature. J. Immunol. Methods. 214:165-174.

Nibbering, P. H., P. C. J. Leijh, and R. Van Furth. 1987. Quantitative immunocytochemical characterization of mononuclear phagocytosis. II. Monocytes and tissue macrophages. Immunology. 62:171-176.

NRC. 1988. Nutrient Requirements of Swine. National Academy Press, Washington, DC.

Nunez, M., J. Bueno, M. Ayudarte, A. Almendros, A. Rios, M. Suarez, and A. Gil. 1996. Dietary restriction induces biochemical and morphometric changes in the small intestine of nursing piglets. J. Nutr. 126:933.

O'Garra, A. and P. Vieira. 2004. Regulatory T cells and mechanisms of immune system control. Nature Medicine 10:801-805.

Parrott, T. D., T. Harbolt, T. Rehberger, B. White, and C.V. Maxwell. 1994. Characterization of the predominate lactobacilli isolated from the gastrointestinal tract of post-weaned pigs. Abstract from the 93rd General Meeting of the American Society of Microbiology, Las Vegas, NV.

Parrott, T.D., T. Rehberger and M. Holt. 2001. Molecular typing of hemolytic *Escherichia coli* isolated from swine. J. Anim. Sci. 79 (Suppl. 1):455.

Piccirillo, C. A. and A. M. Thornton. 2004. Cornerstone of peripheral tolerance: naturally occurring $CD4^+CD25^+$ regulatory T cells. TRENDS in Immunol. 25:374-380.

Pichler, J. W. and T. Wyss-Coray. 1994. T cells as antigen-presenting cells. Immunol. Today 15:312.

Poussier, P. and M. H., Julius. 1997. Preparation of mononuclear cells from the gut-associated immune system. In: Immunology Methods Manual. Academic Press, San Diego, pp. 1500-1520.

Sharma, R., U. Schumacher, V. Ronaasen, and M. Coates. 1995. Rat intestinal mucosal responses to a microbial flora and different diets. Gut 36:209.

Solano-Aguilar, G. I., K. G. Vengroski, E. Beshah, and J. K. Lunney. 2000. Isolation and purification of lymphocyte subsets from gut-associated lymphoid tissue in neonatal swine. J. Immunol. Meth. 241:185-199.

Specian, R. D., and M. G. Oliver. 1991. Functional biology of intestinal goblet cells. Am. J. Physiol. 260:C183.

Spurlock, M. E. 1997. Regulation of metabolism and growth during immune challenge: an overview of cytokine function. J. Anim. Sci. 75:1773-1783.

Tang, M., B. Laarveld, A. G. Van Kessel, D. L. Hamilton, A. Estrada, and J. F. Patience. 1999. Effect of segregated early weaning on postweaning small intestinal development in pigs. J. Anim. Sci. 77:3191.

Thompson, C. and F. Powrie. 2004. Regulatory T cells. Current Opinion in Pharmacol. 4:408-414.

Todd, D., A. J. Singh,D. L. Greiner, J. P. Mordes, A. A. Rossin, and R. Bortell. 1999. A new isolation method for rat intrepithelial lymphocytes. J. Immunol. Methods. 224:111-127.

van Den Bogaard, A. E., M. Hazen, M. Hoyer, P. Oostenbach, and E. E. Stobberingh. 2002. Effects of flavophospholipol on resistance in fecal *Escherichia coli* and Enterococci of fattening pigs. Antimicrob. Agents Chemother. 46:110-118.

Vega-Lopez, M. A., E. Telemo, M. Bailey, K. Stevens, and C. R. Stokes. 1993. Immune cell distributionin the small intestine of the pig: immunohistological evidence for an organized compartmentalization in the lamina propria. Vet. Immunol. Immunopathol. 37:49-60.

Yang, H., J. Lopez, C. Risley, T. Radke and D. Holzgraefe. 2003. Effect of adding abacillus based direct fed microbial on performance of nursery pigs fed diets with or without antibiotics. J. Anim. Sci.

Zoetendal, E. G., B. Cheng, S. Koike, and R. I. Mackie. 2004a. Molecular microbial ecology of the gastrointestinal tract: from phylogeny to function. Curr. Issues Intest. Microbiol. 5:31-47.

Zoetendal, E. G., C. T. Collier, S. Koike, R. I. Mackie, and H. R. Gaskins. 2004b. Molecular ecological analysis of the gastrointestinal microbiota: a review. J. Nutr. 134:465-472.

Table 1. Composition of experimental phase 1 diets (d 0 to14 post-weaning).

Item, %	Phase 1 diets		
	Control	*Bacillus*	Antibiotic
Yellow Corn	44.25	44.00	44.00
Steam rolled oats	5.00	5.00	5.00
Whey	6.00	6.00	6.00
Lactose%	11.00	11.00	11.00
Pro. Soy Prot. (Optipro)	7.45	7.45	7.45
Soybean meal, 48% CP	7.63	7.63	7.63
Plasma Protein (MP-722)	5.00	5.00	5.00
Cells Spray Dried, AP-301	1.75	1.75	1.75
Select menhaden fish meal	4.50	4.50	4.50
Soybean oil	4.00	4.00	4.00
Ethoxyquin	0.03	0.03	0.03
Lysine-HCl	0.16	0.16	0.16
Methionine	0.13	0.13	0.13
Bacillus culture[a]	0.00	0.25	0.00
Antibiotic (Carbadox)[b]	0.00	0.00	0.25
Mineral Premix (NB-8534)[c]	0.15	0.15	0.15
Isoleucine, 85%	0.10	0.10	0.10
Vitamin Premix (NB-6157C)[d]	0.20	0.20	0.20
Dicalcium Phosphate	1.50	1.50	1.50
Calcium carbonate	0.57	0.57	0.57
Threonine	0.08	0.08	0.08
Salt	0.50	0.50	0.50
Calculated Composition, %			
Lysine	1.50	1.50	1.50
Met + Cys	0.85	0.85	0.85
Threonine	0.97	0.97	0.97
Tryptophan	0.27	0.27	0.27
Isoleucine	0.85	0.85	0.85
Ca	0.90	0.90	0.90
Avail. P	0.57	0.57	0.57
Lactose	14.91	14.91	14.91
Metabolizable Energy, kcal/lb	1,550.25	1,546.36	1,546.36

[a]Provided 7.5 x 10^5 cfu/g of feed.
[b]Provided at 50 g per ton.
[c]Supplied 0.30 mg of Se as sodium selenite, 40 mg of Mn as manganous oxide, 165 mg of Zn as zinc oxide, 165 mg of Fe as ferrous sulfate, 17 mg of Cu as copper sulfate, and 0.30 mg of I as calcium iodate per kilogram of feed.
[d]Supplied 11023 IU of vitamin A, 1653 IU of vitamin D_3 as D-activated animal sterol, 44 IU of vitamin E, 4.4 mg of vitamin K as menadione sodium bisulfite complex, 33 mg of pantothenic acid as D-calcium pantothenate, 55 mg niacin, 10 mg of riboflavin, and 44 μg of vitamin B_{12} per kilogram of feed.

Table 2. Composition of experimental phase 2 diets (d 14 to 28 post-weaning).

Item, %	Phase 2 diets		
	Control	*Bacillus*	Antibiotic
Yellow Corn	49.58	49.33	49.33
Lactose%	8.10	8.10	8.10
Soybean meal, 48% CP	30.50	30.50	30.50
Plasma Protein (MP-722)	1.00	1.00	1.00
Cells Spray Dried, AP-301	1.50	1.50	1.50
Select menhaden fish meal	2.00	2.00	2.00
Fat, Darling	4.00	4.00	4.00
Ethoxyquin	0.03	0.03	0.03
Lysine-HCl	0.14	0.14	0.14
Methionine	0.11	0.11	0.11
Threonine	0.08	0.08	0.08
Bacillus culture[a]	0.00	0.25	0.00
Antibiotic (Carbadox)[b]	0.00	0.00	0.25
Mineral Premix (NB-8534)[c]	0.15	0.15	0.15
Isoleucine, 85%	0.00	0.00	0.00
Vitamin Premix (NB-6157C)[d]	0.25	0.25	0.25
Dicalcium Phosphate	1.30	1.30	1.30
Calcium carbonate	0.76	0.76	0.76
Salt	0.50	0.50	0.50
Calculated Composition, %			
Lysine	1.45	1.45	1.45
Met + Cys	0.82	0.82	0.82
Threonine	0.94	0.94	0.94
Tryptophan	0.28	0.28	0.28
Isoleucine	0.88	0.88	0.88
Ca	0.80	0.80	0.80
Avail. P	0.38	0.38	0.38
Lactose	8.02	8.02	8.02
Metabolizable Energy, kcal/lb	1,572.16	1,568.27	1,568.27

[a] Provided 7.5 x 10^5 cfu/g of feed.
[b]Provided at 50 g per ton.
[c]Supplied 0.30 mg of Se as sodium selenite, 40 mg of Mn as manganous oxide, 165 mg of Zn as zinc oxide, 165 mg of Fe as ferrous sulfate, 17 mg of Cu as copper sulfate, and 0.30 mg of I as calcium iodate per kilogram of feed.
[d]Supplied 11023 IU of vitamin A, 1653 IU of vitamin D_3 as D-activated animal sterol, 44 IU of vitamin E, 4.4 mg of vitamin K as menadione sodium bisulfite complex, 33 mg of pantothenic acid as D-calcium pantothenate, 55 mg niacin, 10 mg of riboflavin, and 44 μg of vitamin B_{12} per kilogram of feed.

Table 3. Composition of experimental phase 3 diets (d 28 to 38 post-weaning).

Item, %	Phase 3 diets		
	Control	*Bacillus*	Antibiotic
Yellow Corn	61.85	61.60	61.60
Soybean meal, 48% CP	30.50	30.50	30.50
Fat, Darling	4.00	4.00	4.00
Ethoxyquin	0.03	0.03	0.03
Lysine-HCl	0.15	0.15	0.15
Methionine	0.03	0.03	0.03
Bacillus culture[a]	0.00	0.25	0.00
Antibiotic (Carbadox)[b]	0.00	0.00	0.25
Mineral Premix (NB-8534)[c]	0.15	0.15	0.15
Vitamin Premix (NB-6157C)[d]	0.25	0.25	0.25
Dicalcium Phosphate	1.70	1.70	1.70
Calcium carbonate	0.80	0.80	0.80
Threonine	0.04	0.04	0.04
Salt	0.50	0.50	0.50
Calculated Composition, %			
Lysine	1.20	1.20	1.20
Met + Cys	0.68	0.68	0.68
Threonine	0.78	0.78	0.78
Tryptophan	0.24	0.24	0.24
Isoleucine	0.83	0.83	0.83
Ca	0.80	0.80	0.80
Avail. P	0.38	0.38	0.38
Metabolizable Energy, kcal/lb	1,566.83	1,562.94	1,562.94

[a] Provided 7.5 x 10^5 cfu/g of feed.
[b]Provided at 50 g per ton.
[c]Supplied 0.30 mg of Se as sodium selenite, 40 mg of Mn as manganous oxide, 165 mg of Zn as zinco oxide, 165 mg of Fe as ferrous sulfate, 17 mg of Cu as copper sulfate, and 0.30 mg of I as calcium iodate per kilogram of feed.
[d]Supplied 11023 IU of vitamin A, 1653 IU of vitamin D_3 as D-activated animal sterol, 44 IU of vitamin E, 4.4 mg of vitamin K as menadione sodium bisulfite complex, 33 mg of panthothenic acid as D-calcium pantothenate, 55 mg niacin, 10 mg of riboflavin, and 44 μg of vitamin B_{12} per kilogram of feed.

Table 4. Effect of *Lactobacillus brevis* 1E1 supplementation on average daily gain (ADG) and body weight (BW) of pigs during the pre-weaning period.

	Pre-weaning treatment		
Item	Control	1E1	P-value
ADG, g			
Birth to day 5	181±11	172±12	0.58
Birth to day 10	242±10	234±11	0.58
Birth to day 14	269±9	257±10	0.38
Birth to weaning	297±8	291±9	0.63
BW, kg			
Birth	1.49±.05	1.51±0.05	0.73
Day 5	2.45±0.07	2.42±0.08	0.83
Day 10	3.97±0.11	3.92±0.13	0.78
Day 14	5.32±0.15	5.18±0.16	0.55
Weaning[a]	8.00±0.20	7.92±0.22	0.78
No. born alive	10.94±0.50	11.46±0.57	0.49
No. pigs weaned	10.19±0.58	10.46±0.65	0.76
Survival to weaning, %	92.53±2.92	91.40±3.24	0.80

[a] Adjusted for age at weaning (average age at weaning = 21.77 with a standard deviation of 1.55).

Table 5. Effect of *Lactobacillus brevis* 1E1 administration on average daily gain (ADG), average daily feed intake (ADFI), gain:feed (G:F) and body weight (BW)of pigs fed during the post-weaning period.

	Post-weaning treatment			
Item	Control	1E1	SE	P-value
ADG, kg				
Phase 1	0.092	0.100	0.008	0.51
Phase 2	0.286	0.332	0.013	**0.01**
Phase 3	0.507	0.535	0.017	0.26
Overall (1-3)	0.307	0.336	0.010	**0.05**
ADFI, kg				
Phase 1	0.125	0.128	0.001	0.13
Phase 2	0.304	0.347	0.022	0.17
Phase 3	0.563	0.622	0.018	**0.03**
Overall (1-3)	0.343	0.380	0.012	**0.04**
Gain:Feed				
Phase 1	0.739	0.785	0.069	0.63
Phase 2	0.931	0.988	0.041	0.33
Phase 3	0.931	0.859	0.029	**0.09**
Overall (1-3)	0.882	0.884	0.020	0.93
Weight, kg				
Phase 1	8.73	8.88	0.09	0.24
Phase 2	12.72	13.55	0.22	**0.01**
Phase 3	19.87	21.45	0.37	**< 0.01**

Table 6. Average daily gain (ADG), average daily feed intake (ADFI), gain:feed (G:F) and body weight (BW) of pigs fed control, *Bacillus* and antibiotic in the diet during the nursery period.

	Post- weaning treatment				
Item	Control	*Bacillus*	Antibiotic	SE	P-value
ADG, kg					
Phase 1	0.083	0.098	0.108	0.010	0.21
Phase 2	0.291[b]	0.262[b]	0.374[a]	0.015	**< 0.01**
Phase 3	0.513	0.508	0.541	0.021	0.51
Phase 1-3	0.310[b]	0.299[b]	0.356[a]	0.012	**< 0.01**
ADFI, kg					
Phase 1	0.128	0.125	0.126	0.001	0.12
Phase 2	0.310	0.323	0.344	0.027	0.66
Phase 3	0.581[b]	0.521[b]	0.675[a]	0.022	**< 0.01**
Phase 1-3	0.353[b]	0.334[b]	0.397[a]	0.015	**0.02**
Gain:Feed					
Phase 1	0.641	0.787	0.858	0.086	0.18
Phase 2	0.931[ab]	0.879[b]	1.069[a]	0.050	**0.03**
Phase 3	0.883[b]	0.998[a]	0.805[b]	0.035	**< 0.01**
Phase 1-3	0.870	0.886	0.892	0.025	0.80
Weight, kg					
Initial	7.87	7.86	7.87	0.01	0.92
Phase 1	8.70	8.77	8.95	0.11	0.24
Phase 2	12.70[b]	12.58[b]	14.14[a]	0.27	**< 0.01**
Phase 3	19.99[b]	19.96[b]	22.03[a]	0.46	**< 0.01**

[a,b] Means within a row with different superscripts differ, $P < 0.05$).

Table 7. Adjusted (log 10) *Escherichia coli* and coliform enumeration within the duodenum, jejunum, and ileum of nursery pigs on d 10, 20, and 38 after weaning.

	Sampling day (post-weaning)				
	10	20	38	SE	P=
Duodenum					
E. coli	2.37^{y}	3.92^{x}	2.24^{y}	0.43	0.01
Coliform	3.22^{x,y}	4.00^{x}	2.25^{y}	0.46	0.03
Jejunum					
E. coli	2.50^{y}	4.06^{x}	2.00^{y}	0.31	>0.01
Coliform	3.13^{x,y}	4.07^{x}	2.00^{y}	0.42	>0.01
Ileum					
E. coli	4.05	5.18	4.40	0.56	0.32
Coliform	5.93	5.43	5.22	0.61	0.68

^{x,y} Means within a row with different superscripts differ, P < 0.05).

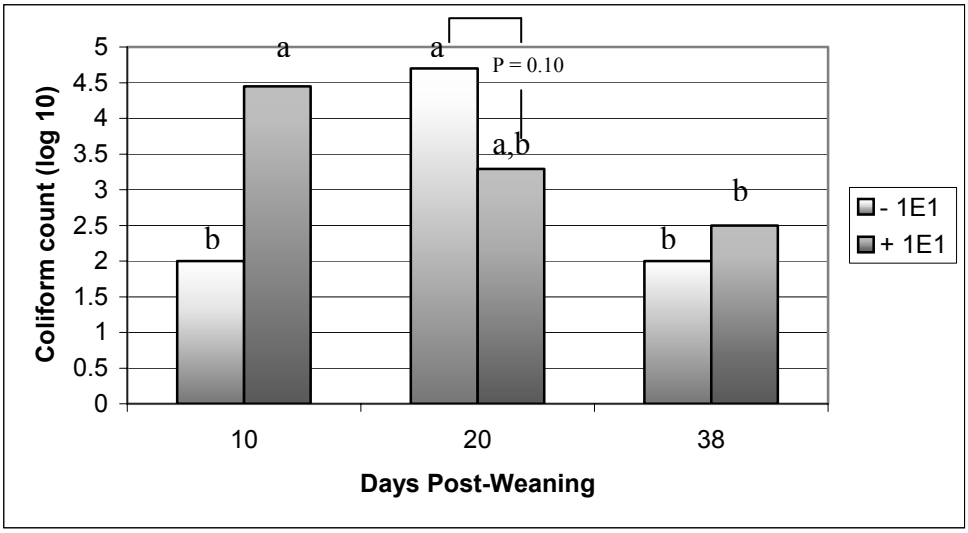


Figure 1. Effect of *Lactobacillus brevis* 1E1 administration on adjusted coliform counts (log 10) within the duodenum of nursery pigs on d 10, 20, and 38 after weaning (1E1 x day interaction, P < 0.05; ^{a,b}P < 0.05).

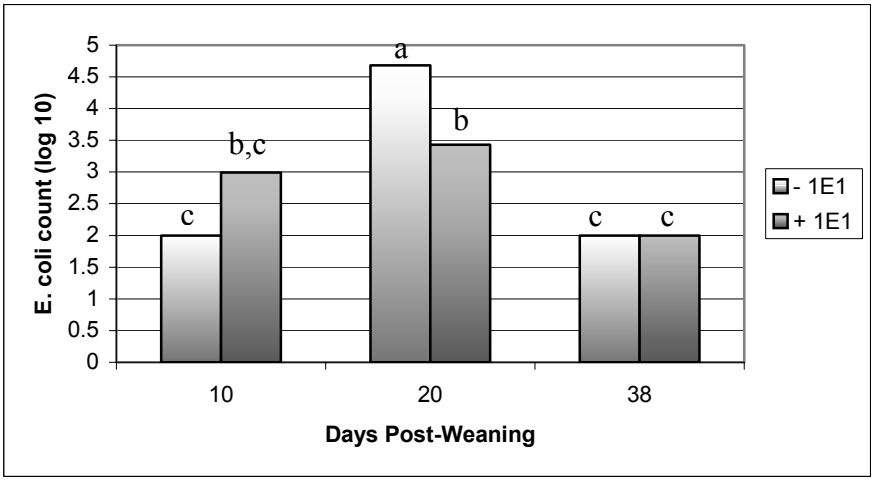


Figure 2. Effect of *Lactobacillus brevis* 1E1 administration on adjusted *Escherichia coli* counts (log 10) within the jejunum of nursery pigs on d 10, 20, and 38 after weaning (1E1 x day interaction, $P < 0.05$; $^{a,b,c}P < 0.05$).

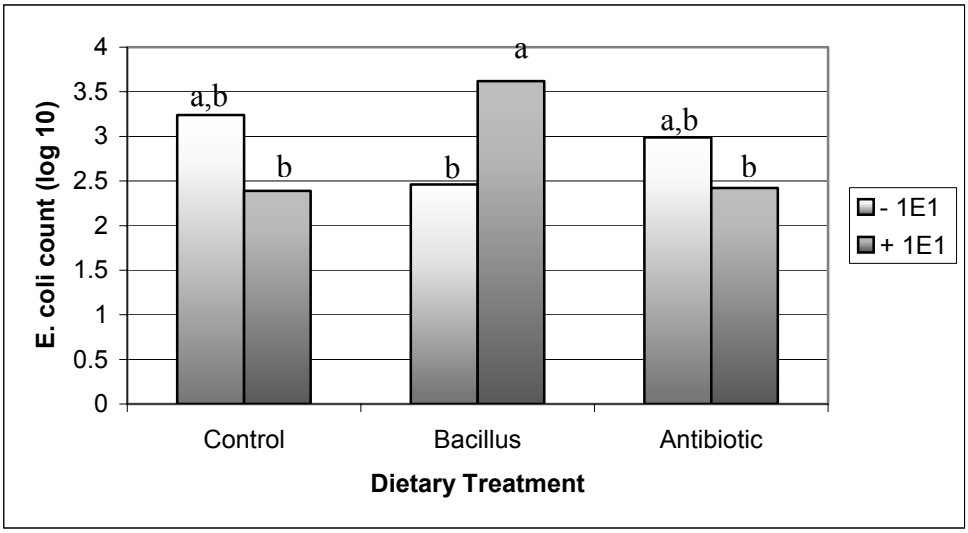
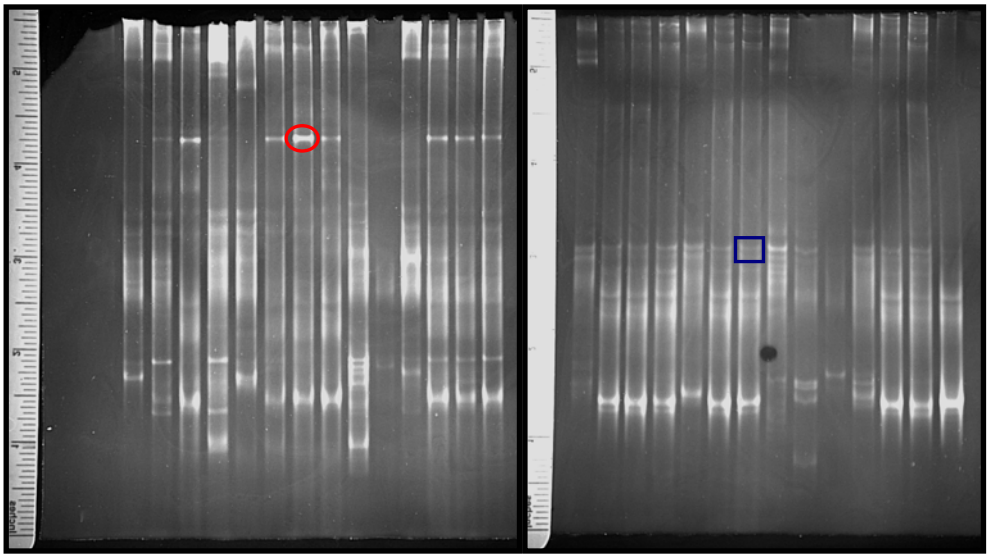


Figure 3. Effect of *Lactobacillus brevis* 1E1 administration and dietary treatment during the nursery phase on adjusted *Escherichia coli* counts (log 10) within the jejunum of nursery pigs (1E1 x nursery diet interaction, $P = 0.05$; $^{a,b}P < 0.06$).



A. B.

Figure 4. Denaturing gradient gels showing bands primarily present in the jejunal samples of pigs A). fed 1E1 (circled band) or B). fed no 1E1 (boxed band).

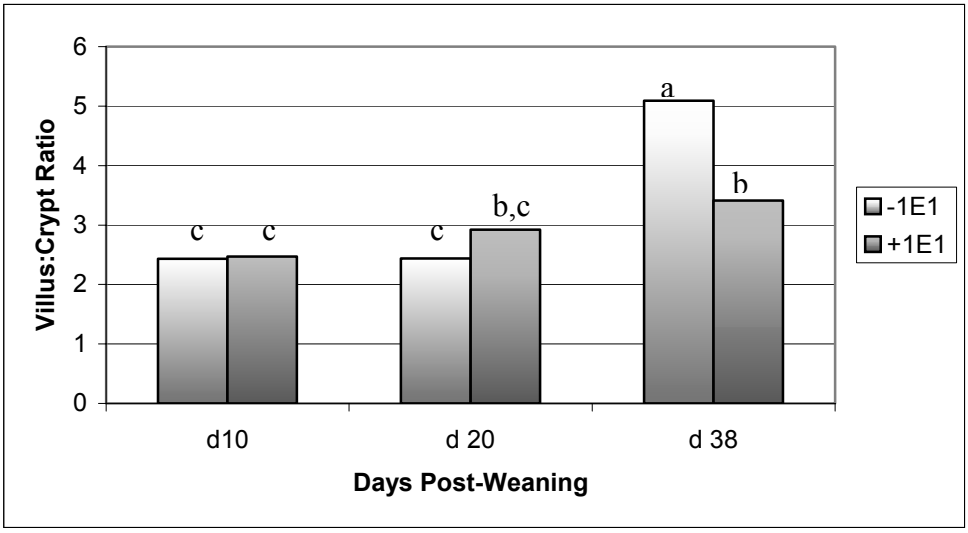


Figure 5. Effect of *Lactobacillus brevis* 1E1 on the villus height:crypt depth ratio within the duodenum of pigs on d 10, 20, and 38 after weaning (1E1 x day interaction, $P < 0.01$; $^{a,b,c}P < 0.05$).

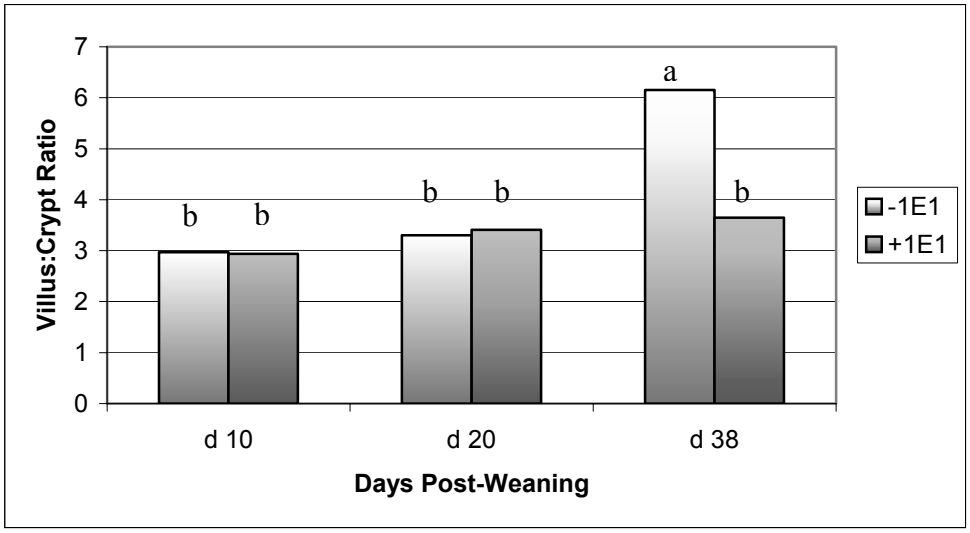


Figure 6. Effect of *Lactobacillus brevis* 1E1 on the villus height:crypt depth ratio within the jejunum of pigs on d 10, 20, and 38 after weaning (1E1 x day interaction, $P < 0.01$; $^{a,b}P < 0.05$).

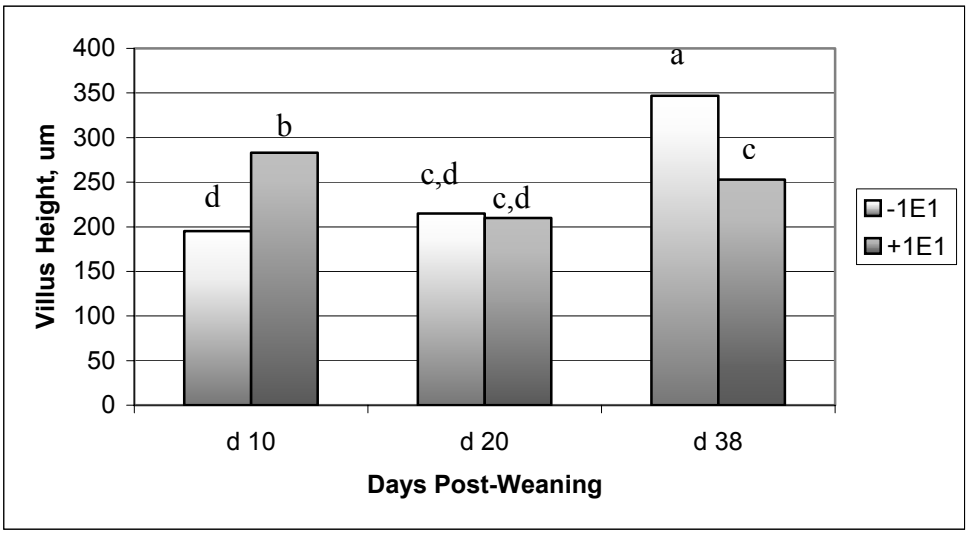


Figure 7. Effect of *Lactobacillus brevis* 1E1 on the villus height within the ileum of pigs on d 10, 20, and 38 after weaning (1E1 x day interaction, $P < 0.01$; $^{a,b,c,d}P < 0.05$).

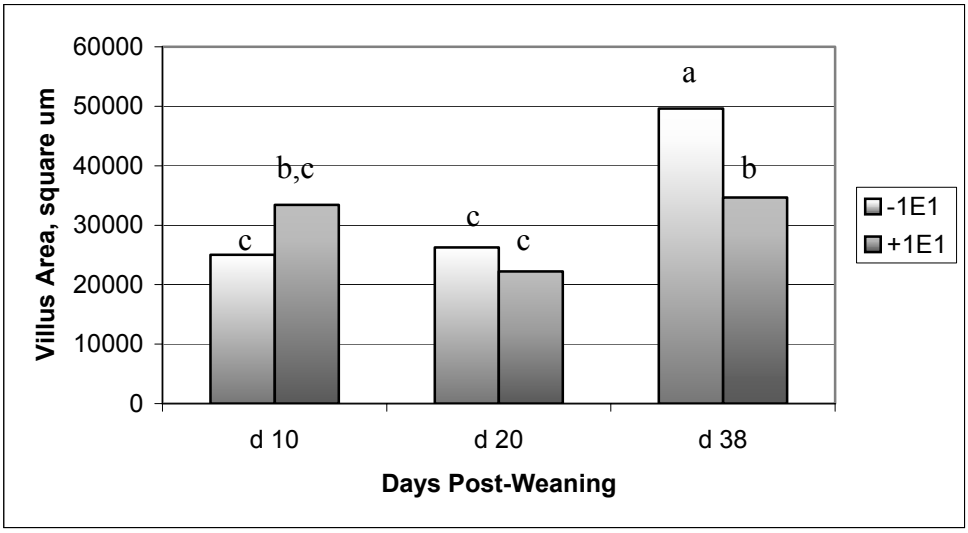


Figure 8. Effect of *Lactobacillus brevis* 1E1 on the villus area within the ileum of pigs on d 10, 20, and 38 after weaning (1E1 x day interaction, $P < 0.01$; $^{a,b,c}P < 0.05$).

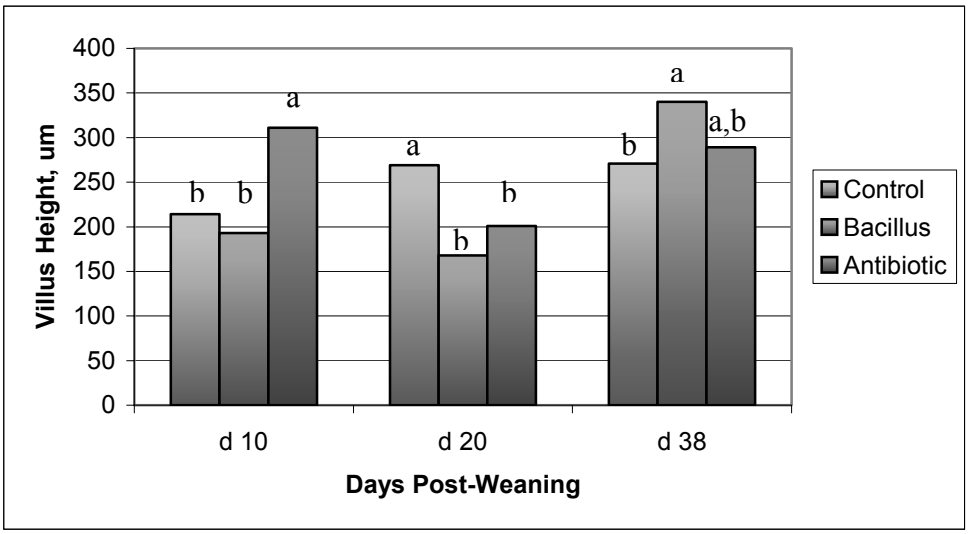


Figure 9. Effect of dietary nursery treatment on the villus height within the ileum of pigs on d 10, 20, and 38 after weaning (nursery diet x day interaction, $P < 0.01$; ^{a,b} Means within each day with differing letter are significantly different, $P < 0.05$).

Table 8. Effect of *Lactobacillus brevis* (1E1) and dietary treatments during the nursery period on neutral, acidic, and sulfuric goblet cell enumeration in the duodenum, jejunum, and ileum of the pig small intestine.

		Lactobacillus brevis (1E1)			Nursery Diet			
Tissue	Goblet cell type	-	+	SE	Control	*Bacillus*	Antibiotic	SE
Duodenum	Neutral	3.02	2.56	0.64	2.70	2.39	3.28	0.80
	Acidic	15.26 [b]	22.85 [a]	1.04	19.09	19.99	18.09	1.29
	Sulfuric	19.97 [a]	10.94 [b]	0.95	18.95 [a]	13.19 [b]	14.23 [b]	1.19
Jejunum	Neutral	3.13	2.67	0.35	3.69	2.08	2.93	0.44
	Acidic	12.00 [b]	22.33 [a]	1.09	16.65	18.67	16.18	1.35
	Sulfuric[1]	18.52 [a]	10.88 [b]	0.71	17.76 [a]	12.57 [b]	13.77 [b]	0.88
Ileum	Neutral	4.38	4.96	0.70	5.27	4.95	3.79	0.87
	Acidic[2]	13.90	20.57	0.90	17.87	17.64	16.20	1.12
	Sulfuric[3]	20.80	11.76	1.03	20.61	12.84	15.40	1.28

[a,b] Means in a row within a treatment with no letters in common differ (P < 0.05).
[1] Nursery diet x day interaction, P < 0.05; See Figure 10.
[2] 1E1 x day interaction, P < 0.01; see Figure 11.

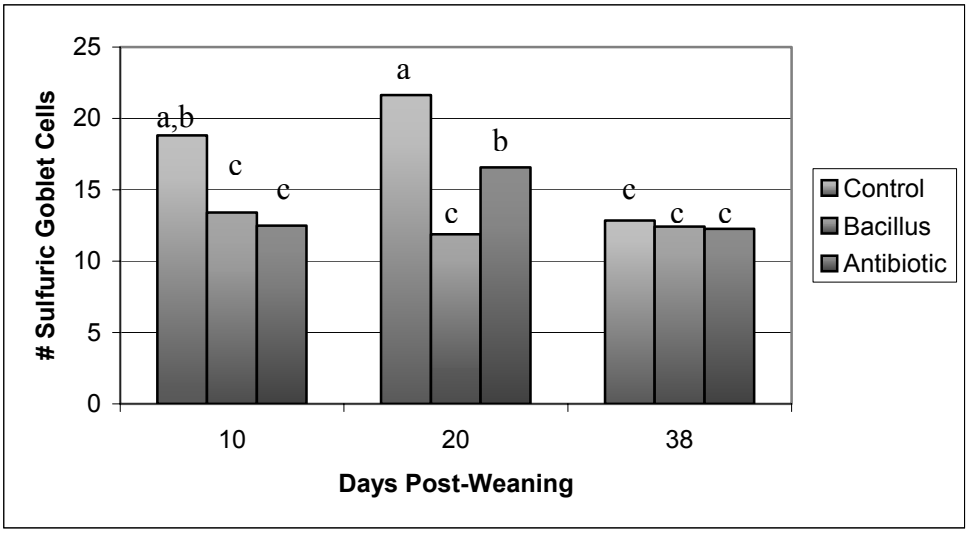


Figure 10. Effect of dietary treatments during the nursery period on the enumeration of sulfated mucin-producing (sulfuric) goblet cells in the jejunum of the pig small intestine (nursery diet x day interaction, P < 0.01; [a,b,c] P < 0.05).

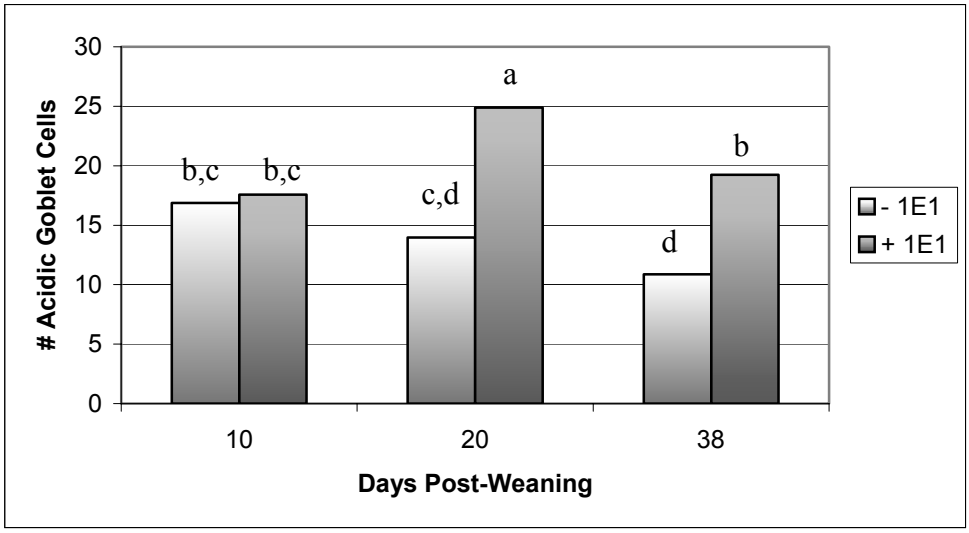


Figure 11. Effect of *Lactobacillus brevis* (1E1) on the enumeration of acidic sialylated goblet cells in the ileum of the pig small intestine (1E1 x day interaction, $P < 0.01$; ^{a,b,c,d} $P < 0.05$).

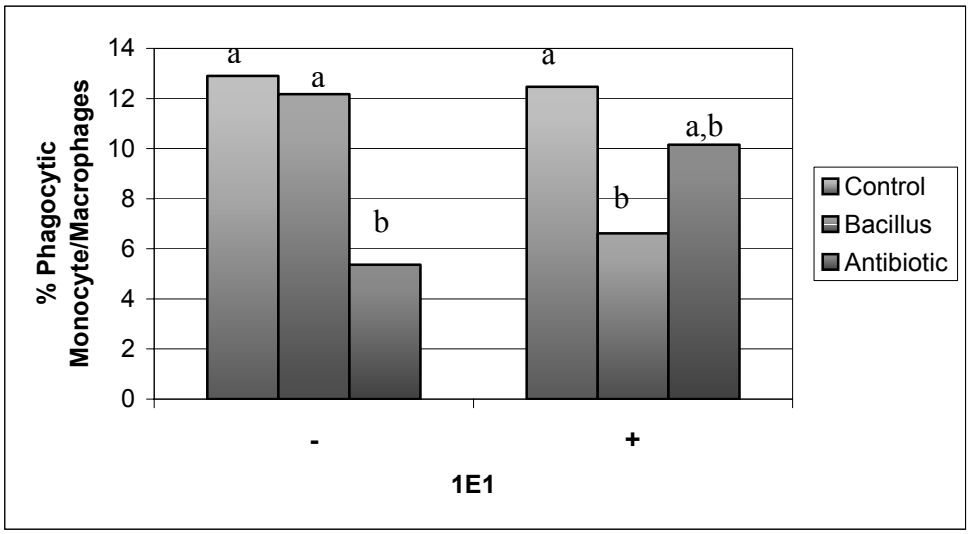


Figure 12. Effect of *Lactobacillus brevis* 1E1 administration and dietary treatments fed during the nursery period on the percentage of monocyte/macrophages isolated from the peripheral blood of pigs during the post-weaning period (1E1 x diet interaction, $P < 0.10$; $^{a,b}P < 0.09$).

Table 9. Main effects of 1E1 administration and dietary treatments during the nursery period on the average number of sheep red blood cells consumed by phagocytic monocyte/macrophages isolated from the peripheral blood of pigs post-weaning.

	1E1		
Treatment:	-	+	P=
SRBC consumed	1.28±0.16	1.44±0.17	0.49

	Dietary Treatment			
Treatment:	Control	*Bacillus*	Antibiotic	P=
SRBC consumed	1.36±0.21^{x,y}	1.68±0.20^{x}	1.04±0.19^{y}	0.10

^{x,y} Means within a row with different superscripts are significantly different; P < 0.05).

Table 10. Main effects of 1E1 administration and dietary treatments during the nursery period on the number of cells/villi expressing various cell surface markers within the jejunum of pigs.

	1E1		
Treatment:	-	+	P=
Cell surface marker			
CD4	2.35 ± 0.72	2.10 ± 0.69	0.80
CD8	1.21 ± 0.33	0.73 ± 0.31	0.30
CD25	2.16 ± 0.54	1.94 ± 0.51	0.76
MHCII	1.80 ± 0.62	2.11 ± 0.59	0.72

	Dietary Treatment			
Treatment:	Control	*Bacillus*	Antibiotic	P=
Cell surface marker				
CD4	2.22 ± 0.82	2.41 ± 0.93	2.06 ± 0.84	0.87
CD8	1.36 ± 0.37	0.92 ± 0.42	0.64 ± 0.38	0.41
CD25	2.61 ± 0.61	1.69 ± 0.70	1.85 ± 0.62	0.56
MHCII	2.28 ± 0.70	1.25 ± 0.80	2.34 ± 0.72	0.54

Table 11. Effect of *Lactobacillus brevis* (1E1) and dietary treatments during the nursery period on the proportion of peripheral blood mononuclear cells expressing CD3, CD4, CD8, CD21, CD25, TCR1, and MHC-II cell surface molecules.

Cell surface marker	Day post-weaning	*Lactobacillus brevis* (1E1)		Nursery Diet		
		-	+	Control	*Bacillus*	Antibiotic
CD3*	10	4.46 ± 2.67	11.75 ± 2.79	9.54 ± 3.62	9.67 ± 3.41	5.11 ± 2.96
	20	17.63 ± 2.41	24.33 ± 2.41	21.86 ± 2.96	14.20 ± 2.96	26.87 ± 2.96
	38	26.10 ± 2.41	24.21 ± 2.41	19.83 ± 2.96	34.81 ± 2.96	20.82 ± 2.96
MHC-II*	10	14.62 ± 3.01	13.76 ± 3.15	16.20 ± 4.09	17.37 ± 3.85	9.00 ± 3.34
	20	38.92 ± 2.72	48.51 ± 2.72	27.08 ± 3.34	49.26 ± 3.34	54.80 ± 3.34
	38	40.94 ± 2.72	40.39 ± 2.72	44.17 ± 3.34	39.18 ± 3.34	38.64 ± 3.34
CD4[1]	10	2.71 ± 1.53	3.87 ± 1.37	**4.06 ± 1.78^{c,d}**	**3.86 ± 1.78^{c,d}**	**1.95 ± 1.78^{d}**
	20	13.39 ± 1.19	13.84 ± 1.19	**9.24 ± 1.46^{b,c}**	**15.64 ± 1.46^{a}**	**15.98 ± 1.46^{a}**
	38	12.06 ± 1.19	10.87 ± 1.19	**12.91 ± 1.46^{a,b}**	**13.17 ± 1.45^{a}**	**8.31 ± 1.46^{c}**
CD8*	10	4.28 ± 2.00	9.21 ± 2.09	3.63 ± 2.72	10.06 ± 2.56	6.53 ± 2.22
	20	28.06 ± 1.81	38.70 ± 1.81	23.92 ± 2.22	35.58 ± 2.22	40.64 ± 2.22
	38	43.67 ± 1.81	38.99 ± 1.81	37.36 ± 2.22	39.79 ± 2.22	46.84 ± 2.22
TCR1*	10	11.45 ± 3.05	15.12 ± 3.19	13.69 ± 4.14	15.01 ± 3.90	11.15 ± 3.38
	20	29.89 ± 2.76	39.55 ± 2.76	31.34 ± 3.38	28.18 ± 3.38	44.64 ± 3.38
	38	29.15 ± 2.76	28.21 ± 2.76	29.08 ± 3.38	31.21 ± 3.38	25.76 ± 3.38
CD25[2]	10	8.46 ± 2.20	9.00 ± 2.29	**10.60 ± 2.98^{c,d}**	**8.89 ± 2.81^{c,d}**	**6.69 ± 2.43^{d}**
	20	22.68 ± 1.99	24.73 ± 1.99	**17.64 ± 2.43^{b,c}**	**26.54 ± 2.43^{a}**	**26.93 ± 2.43^{a}**
	38	17.12 ± 1.99	18.20 ± 2.29	**19.35 ± 2.98^{b}**	**19.38 ± 2.43^{b}**	**14.25 ± 2.43^{c}**
CD21	10	5.52 ± 2.12	6.03 ± 2.22	7.96 ± 2.88	7.07 ± 2.71	2.29 ± 2.35
	20	12.01 ± 1.92	14.27 ± 1.92	8.51 ± 2.35	15.83 ± 2.35	15.08 ± 2.35
	38	10.43 ± 1.92	10.96 ± 1.92	11.15 ± 2.35	11.76 ± 2.35	9.17 ± 2.35

* 1E1 x nursery diet x day interaction, See Figures 13, 15, 16, and 17.

[1] Nursery diet x day interaction for CD4+ lymphocytes, P = 0.02;

[2] Nursery diet x day interaction for CD25+ lymphocytes, P = 0.07;

[a,b,c] Means representing the proportion of PBMC expressing a specific leukocyte cell surface marker within nursery diet treatment with no common superscript differ (P < 0.05).

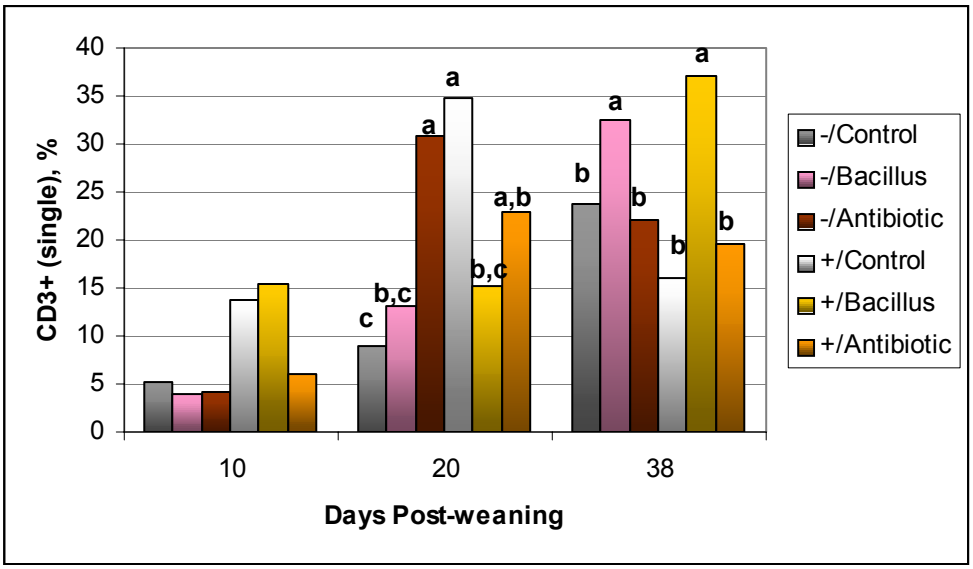


Figure 13. Effect of 1E1 (- or +) and dietary supplementation with *Bacillus* and antibiotic on the proportion of $CD3^+$ lymphocytes within the peripheral blood mononuclear cell population d 10, 20, and 38 post-weaning (1E1 x diet x day interaction, P = 0.03; [a,b,c] Within each day post-weaning, means without a common letter differ, P < 0.05).

Table 12. Effect of *Lactobacillus brevis* (1E1) and dietary treatments during the nursery period on the proportion of peripheral blood mononuclear cells expressing the CD3 and MHC-II cell surface molecules.

Cell surface marker	Day post-weaning	*Lactobacillus brevis* (1E1)		Nursery Diet		
		-	+	Control	*Bacillus*	Antibiotic
$CD3^+MHCII^-$	10	2.11 ± 1.06	2.26 ± 1.11	2.58 ± 1.44	2.80 ± 1.36	1.18 ± 1.18
	20	1.35 ± 0.96	0.99 ± 0.96	1.86 ± 1.18	0.92 ± 1.18	0.72 ± 1.18
	38	0.98 ± 0.96	1.17 ± 0.96	1.14 ± 1.18	1.62 ± 1.18	0.46 ± 1.18
$CD3^+MHCII^+$ *	10	5.83 ± 2.75	11.07 ± 2.88	10.42 ± 3.74	9.06 ± 3.52	5.89 ± 3.05
	20	19.79 ± 2.49	29.01 ± 2.49	23.95 ± 3.05	16.96 ± 3.05	32.29 ± 3.05
	38	22.52 ± 2.49	22.82 ± 2.49	15.31 ± 3.05	34.17 ± 3.05	18.54 ± 3.05
$CD3^-MHCII^-$	10	82.69 ± 4.08	71.33 ± 4.27	71.35 ± 5.54	74.39 ± 5.23	85.28 ± 4.53
	20	38.05 ± 3.69	16.24 ± 3.69	46.47 ± 4.53	21.68 ± 4.53	13.28 ± 4.53
	38	31.76 ± 3.69	36.27 ± 3.69	40.20 ± 4.53	21.21 ± 4.53	40.63 ± 4.53
$CD3^-MHCII^+$ [1]	10	9.37 ± 4.00	15.34 ± 4.18	**15.66 ± 5.43[d]**	**13.75 ± 5.12[d]**	**7.65 ± 4.44[d]**
	20	40.82 ± 3.62	53.76 ± 3.62	**27.72 ± 4.44[d]**	**60.45 ± 4.44[a]**	**53.71 ± 4.44[a,b]**
	38	44.75 ± 3.62	39.74 ± 3.62	**43.35 ± 4.44[b,c]**	**43.01 ± 4.44[b,c]**	**40.37 ± 4.44[c]**

* 1E1 x nursery diet x day interaction, See Figure 14 for $CD3^+MHCII^+$ interaction.
[1] Nursery diet x day interaction, P < 0.01; [a,b,c,d] Means representing the proportion of PBMC expressing specific leukocyte cell surface molecules within all days of a treatment with no common superscript differ (P < 0.05).

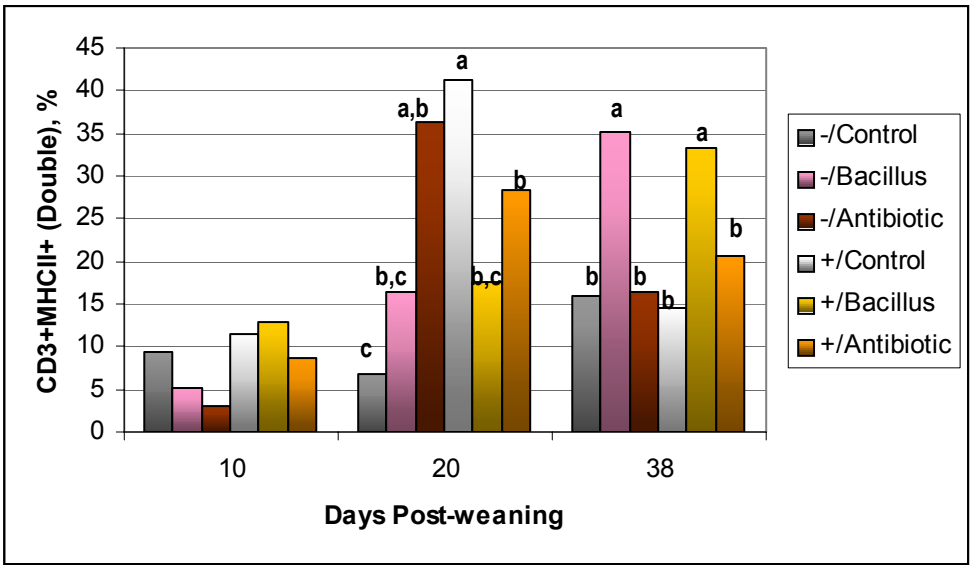


Figure 14. Effect of 1E1 (- or +) and dietary supplementation with *Bacillus* and antibiotic on the proportion of $CD3^{+}MHCII^{+}$ lymphocytes within the peripheral blood mononuclear cell population d 10, 20, and 38 post-weaning (1E1 x diet x day interaction, P = 0.04; [a,b,c] Within each day post-weaning, means without a common letter differ, P < 0.05).

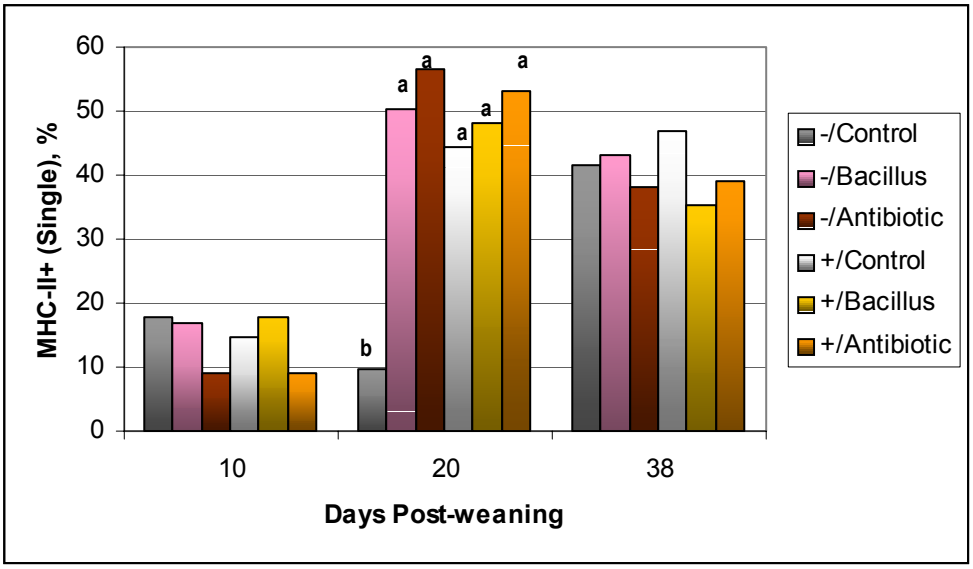


Figure 15. Effect of 1E1 (- or +) and dietary supplementation with *Bacillus* and antibiotic on the proportion of $MHC\text{-}II^{+}$ leukocytes within the peripheral blood mononuclear cell population d 10, 20, and 38 post-weaning (1E1 x diet x day interaction, P = 0.04; [a,b] Within each day post-weaning, means without a common letter differ, P < 0.05).

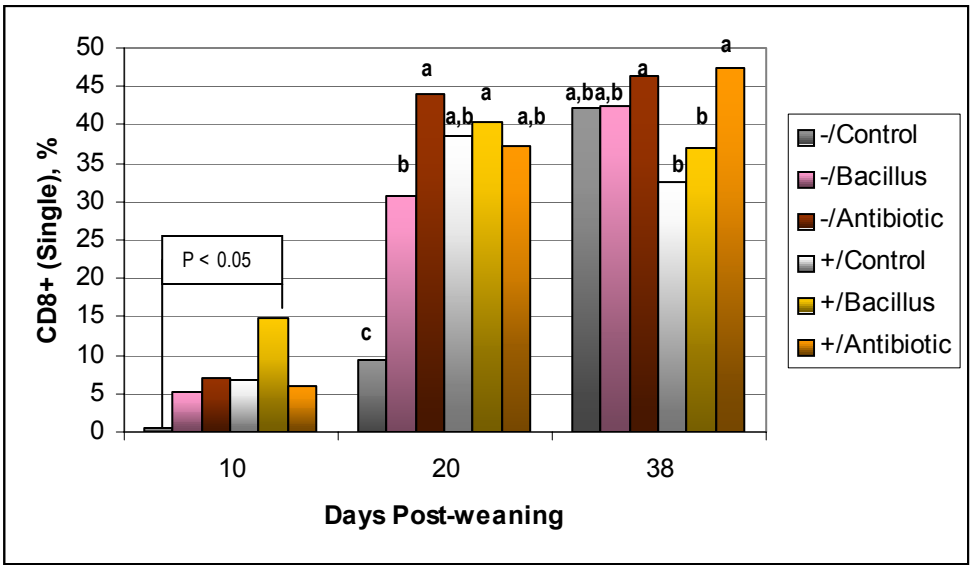


Figure 16. Effect of 1E1 (- or +) and dietary supplementation with *Bacillus* and antibiotic on the proportion of $CD8^+$ lymphocytes within the peripheral blood mononuclear cell population d 10, 20, and 38 post-weaning (1E1 x diet x day interaction, P = 0.001; [a,b,c] Within each day post-weaning, means without a common letter differ, P < 0.05).

Table 13. Effect of *Lactobacillus brevis* (1E1) and dietary treatments during the nursery period on the proportion of peripheral blood mononuclear cells expressing the CD4 and CD8 cell surface molecules.

		Lactobacillus brevis (1E1)		Nursery Diet		
Cell surface marker	Day post-weaning	-	+	Control	*Bacillus*	Antibiotic
$CD4^+CD8^-$	10	5.32 ± 1.83	5.59 ± 1.91	8.76 ± 2.48	4.56 ± 2.34	3.06 ± 2.03
	20	6.18 ± 1.65	6.24 ± 1.65	5.28 ± 2.03	5.74 ± 2.03	7.62 ± 2.03
	38	4.64 ± 1.65	4.08 ± 1.65	5.24 ± 2.03	3.84 ± 2.03	4.00 ± 2.03
$CD4^+CD8^+$	10	1.69 ± 1.20	2.08 ± 1.25	1.93 ± 1.63	2.07 ± 1.53	1.66 ± 1.33
	20	8.61 ± 1.08	10.91 ± 1.08	7.54 ± 1.33	10.34 ± 1.33	11.40 ± 1.33
	38	8.69 ± 1.08	8.30 ± 1.08	8.15 ± 1.33	7.58 ± 1.33	9.76 ± 1.33
$CD4^-CD8^-$ *	10	86.80 ± 3.08	82.35 ± 3.22	82.96 ± 4.18	83.63 ± 3.95	87.12 ± 3.42
	20	66.39 ± 2.79	57.03 ± 2.79	70.17 ± 3.42	60.69 ± 3.42	54.27 ± 3.42
	38	48.08 ± 2.79	53.83 ± 2.79	57.05 ± 3.42	53.41 ± 3.42	42.40 ± 3.42
$CD4^-CD8^+$ [1]	10	**6.18 ± 2.15[d]**	**9.99 ± 2.24[d]**	6.35 ± 2.91	9.74 ± 2.75	8.16 ± 2.38
	20	**18.83 ± 1.94[c]**	**25.82 ± 1.94[b]**	17.01 ± 2.38	23.24 ± 2.38	26.72 ± 3.38
	38	**38.59 ± 1.94[a]**	**33.80 ± 1.94[a]**	29.56 ± 2.38	35.17 ± 2.38	43.84 ± 2.38

* 1E1 x nursery diet x day interaction; P < 0.05.
[1] 1E1 x day interaction, P < 0.01;
[a,b,c,d] Means representing the proportion of PBMC expressing specific leukocyte cell surface molecules within all days of a treatment with no common superscript differ (P < 0.05).

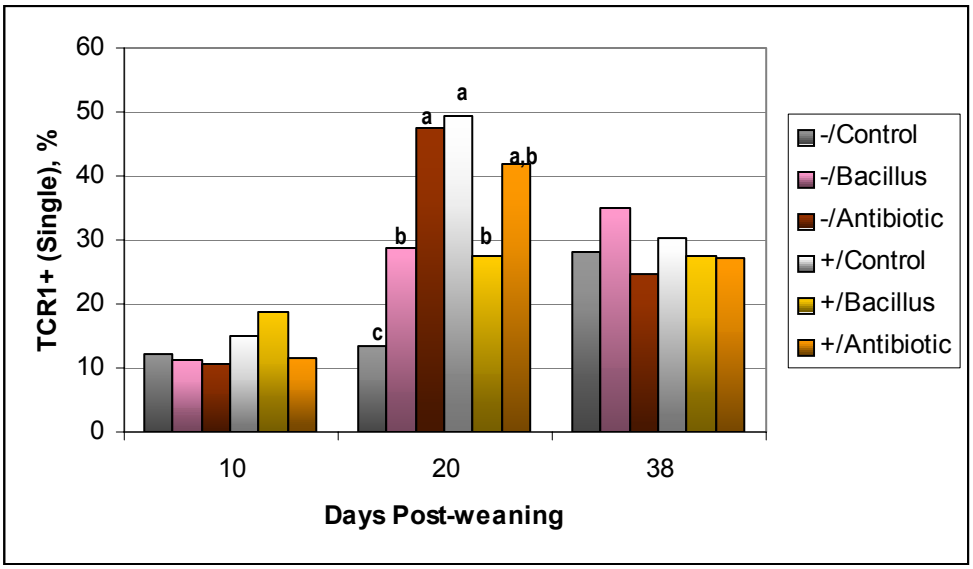


Figure 17. Effect of 1E1 (- or +) and dietary supplementation with *Bacillus* and antibiotic on the proportion of $TCR1^+$ lymphocytes within the peripheral blood mononuclear cell population d 10, 20, and 38 post-weaning (1E1 x diet x day interaction, P = 0.04; ^{a,b,c} Within each day post-weaning, means without a common letter differ, P < 0.05).

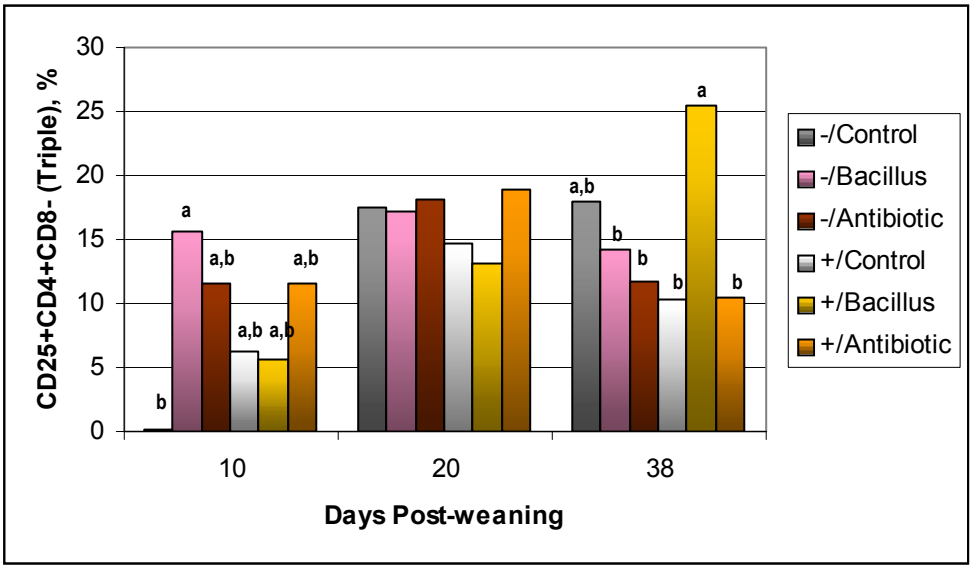


Figure 18. Effect of 1E1 (- or +) and dietary supplementation with *Bacillus* and antibiotic on the proportion of $CD4^+$ lymphocytes within the $CD25^+$ population isolated from peripheral blood on d 10, 20, and 38 post-weaning (1E1 x diet x day interaction, P = 0.04; ^{a,b,c} Within each day post-weaning, means without a common letter differ, P < 0.05).

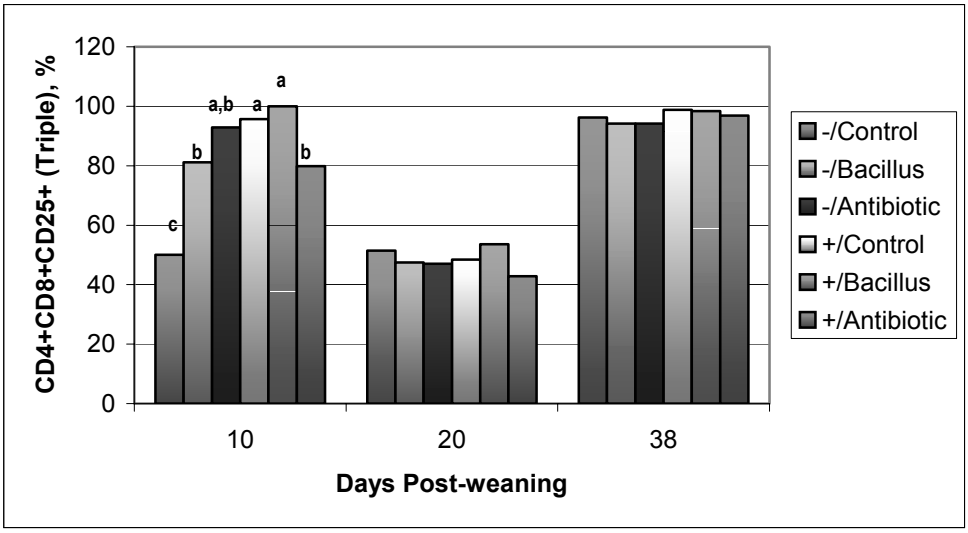


Figure 19. Effect of 1E1 (- or +) and dietary supplementation with *Bacillus* and antibiotic on the proportion of $CD25^+$ lymphocytes within the $CD4^+CD8^+$ population isolated from peripheral blood on d 10, 20, and 38 post-weaning (1E1 x diet x day interaction, P = 0.004; ^{a,b,c} Within each day post-weaning, means without a common letter differ, P < 0.05).