HEALTH OF NON-RUMINANT NEONATES: MANAGEMENT AND GUT MICROBIOTA

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INTRODUCTION

The swine industry has implemented early weaning for efficient and economical pig production (Wilson, 1995; Patience *et al.*, 1997). The obvious consequence of weaning is the abrupt change in diet from sow's milk to solid feed and a change in the environment. The intestinal microflora can be adversely impacted during weaning resulting in higher numbers of potentially pathogenic, acid-intolerant coliforms and a decline of favorable lactobacilli (Bolduan, 1999). In addition, because the piglets are young, their immune system might not be equipped to deal with such pathogenic challenges. The gastrointestinal tract of the pig harbors a metabolically active microbiota that stimulates the normal maturation of host tissues and provides key defense functions (Gaskins, 2001). Several recent examples of improved postweaning performance in the young pig suggest that much of the improvement observed in nutritional studies and in alternative management systems may be through protection of the naïve pig to minimize the impact of rapid changes in gut microbiota in the postweaning pig. Research at the University of Arkansas has attempted to both assess the impact of management system on immune function and performance as well as determine if direct-fed microbials (DFM) and/or signatory molecules from yeast cell wall products have merit as a means of producing an environment in the gastrointestinal tract of the young pig to promote appropriate immune development and ease the postweaning transition faced by the early-weaned pig.

NUTRITION

Although a relatively stable microbiota eventually establishes in the mature pig's digestive tract, it requires a considerable amount of adaptation during the early life periods (Gaskins, 2001). Several recent examples of improved postweaning performance in the young pig suggest that much of the improvement observed in nutritional studies may be through an impact on the intestinal microbiota. Plasma protein has been shown to enhance growth performance in the early growth period of the early-weaned pig (Maxwell and Carter, 2001). Current evidence suggests that it is the globular fraction (Owen et al., 1995) that is responsible for the improved performance and that much of the improvement is lost in pigs reared under high hygiene conditions (Coffey and Cromwell, 1995; Table 1).

Table 1. Effect of nursery environment and protein source on performance of early weaned pigs (d 0 to 14).

	SEW off-site		Conventional on-site	
Protein source	DSM	SDPP	DSM	SDPP
ADG, g[a]	284	300	203	269
ADFI, g[b]	402	470	251	399
Feed:gain[c]	1.53	1.70	1.18	1.34

[a] Protein source x nursery environment interaction, $P < 0.001$.
[b] Protein source x nursery environment interaction, $P < 0.05$.
[c] Dried skim milk (DSM) vs. spray dried plasma protein (SDPP), $P < 0.04$.

Although not demonstrated to date, this suggests that the most likely mechanism of action of plasma proteins is mediated through altering the microbiota or through protecting the pig from exposure to microbial toxins. Our understanding of how one improves pig performance by dietary means has resulted in improved pig performance; however, the dietary approach leads to a population of naïve pigs that become hyper-responders to immune challenges. This is documented in pigs fed plasma protein and subsequently challenged with LPS (Touchette et al., 2002; Carroll et al., 2002; Frank et al., 2003). Cortisol levels are elevated in pigs fed plasma protein and challenged with LPS, indicating that pigs fed plasma protein are more sensitive to endotoxin challenge which results in a situation where hyper-responses can occur. ACTH levels respond in a similar manner with increased responsiveness in pigs fed plasma protein. Similarly, the current concept of the effects of dietary acidification on improved performance suggests that the likely mechanism is via alteration of the intestinal microbiota (Maxwell and Carter, 2001). Finally, research by Hathaway et al. (1999) found that the increase in serum IGF-1 in pigs fed the antibiotic ASP-250 was not due to increased feed intake, suggesting that the effect is mediated through some other mechanism. A likely candidate is via altering the intestinal microbiota or toxin exposure in the pig. These 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).

MANAGEMENT

The usual weaning procedures are accompanied by a general weakening of the immune system (Bolduan, 1999). Early weaning at an age of less than 21 days followed by removal of pigs to a second isolated site is commonly referred to as segregated early weaning (SEW). It reduces the incidence of a number of pathogens, thus reducing immunological stress, which results in improved growth and higher efficiency of feed utilization (Reviewed by Maxwell and Sohn, 1999). This strategy has been successful in reducing the number of pathogens, but has not been successful in eliminating all pathogens. The premise is that pigs are removed from the sow while their immunity, as a consequence of maternal antibodies, is still high. This maternally derived passive immunity will prevent vertical transfer of indigenous pathogens. Pigs reared in isolation have been shown to have reduced immunological stress (Johnson, 1997), resulting in improved growth and efficiency of feed utilization. This is consistent with observations in our research at the University of Arkansas to determine if differences in immune stimulation can

explain performance differences in conventional vs. off-site reared pigs. In the aforementioned study, a total of 432 weanling barrows (19 ± 2 d of age) were obtained from a local commercial company from a single source. Half of the barrows were selected for the off-site nursery study (6 pigs/pen) with the remaining pigs staying in the conventional nursery facilities (approximately18 pigs/pen). Pigs were weighed and serum samples obtained via venipuncture on d 0, 14, and 34 postweaning from a total of 72 pre-selected pigs. Serum α_1-acid glycoprotein concentrations were determined using a commercial kit (porcine α_1-acid glycoprotein plate, Development Technologies International, Inc., Frederick, MD) and a single radial immunodiffusion method. Pigs reared in the off-site nursery were 0.89 kg heavier ($P < 0.01$) at d 14 postweaning and 2.40 kg heavier ($P < 0.01$) at 34 d postweaning. In addition, serum α_1-acid glycoprotein concentration was elevated ($P < 0.01$) in pigs reared in the conventional nursery. This suggests that reduced performance in a conventional nursery may be due to the immunological stress associated with production under these conditions.

To further investigate the differences between conventionally reared pigs (CONV) and segregated early weaned (SEW) pigs, a study was conducted at the University of Arkansas to evaluate the effect of weaning system on growth performance and immune function (Brown et al., 2002b). Growth performance and immune profiles were compared between pigs in CONV and SEW facilities. In addition, we compared the phenotypic expression of surface antigens of isolated lymphocytes from the blood and gastrointestinal tract of pigs reared under CONV and SEW conditions at d 1, 3, 11, and 25 after weaning. Immunohistochemistry procedures were used to compare surface antigens of lymphocytes in the jejunum. During phase 1, ADG ($P < 0.01$) and ADFI ($P < 0.01$) were greater in SEW pigs when compared to pigs reared in CONV facilities, and pigs reared in SEW facilities were heavier at 24 d postweaning ($P < 0.05$) than CONV pigs (17.38 ± 0.35 kg vs. 15.85 ± 0.35 kg, respectively). Neutrophils as a percentage of plasma leucocytes increased ($P < 0.05$) in SEW pigs from d 1 through 3 postweaning (Figure 1; Brown et al., 2002b) followed by an increase ($P < 0.05$) in plasma lymphocytes on d 11 (Figure 2). An increase in CD4+ and CD8+ T cells (Figure 3) and cells positive for the $\gamma\delta$ T cell receptor among jejunal intraepithelial lymphocytes was observed earlier postweaning in the jejunum of pigs reared in the SEW facility compared to CONV pigs (d 1 and 3 postweaning compared to d 3 and 11 postweaning, respectively; Brown et al., 2002a). Immunohistochemistry results indicate that the number of CD4+ and CD8+ T cells per villus expanded more rapidly in the jejunum of SEW pigs when compared to pigs reared in CONV facilities (Brown et al., 2006b). An increased number of CD25+ cells (activated T and B cells) was also observed in the jejunum of SEW pigs on d 3 postweaning. These findings are the opposite of expected results and perhaps suggests that the move to the SEW environment may have exposed piglets to previously un-encountered antigens.

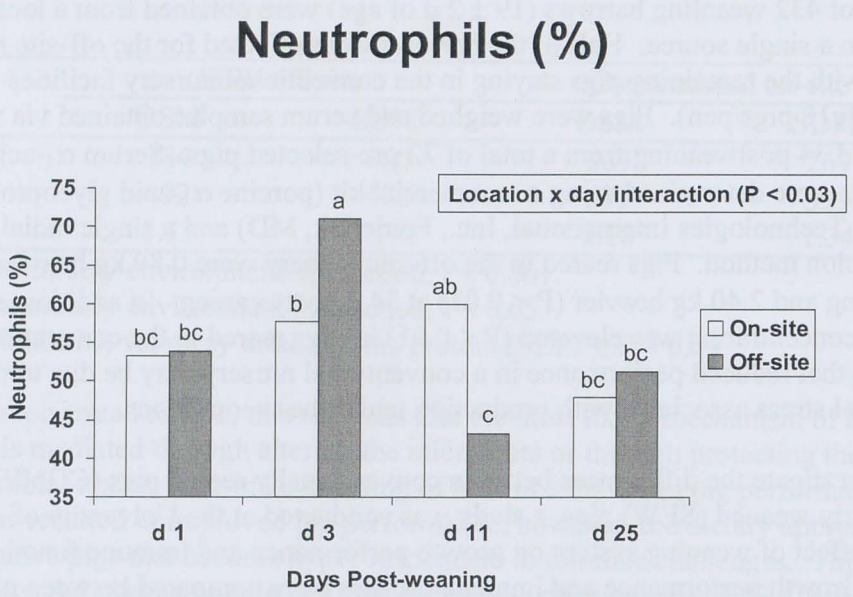


Figure 1. Effect of rearing system on percentage of neutrophils as a percentage of plasma leukocytes on d1, 3, 11 and 25 postweaning. Pigs were weaned at 19 d of age. Values are means of 4 pigs representing each management system for each day after weaning. [a, b, c] Least squares means with a different superscript differs, $P < 0.05$). Brown et al., 2002b.

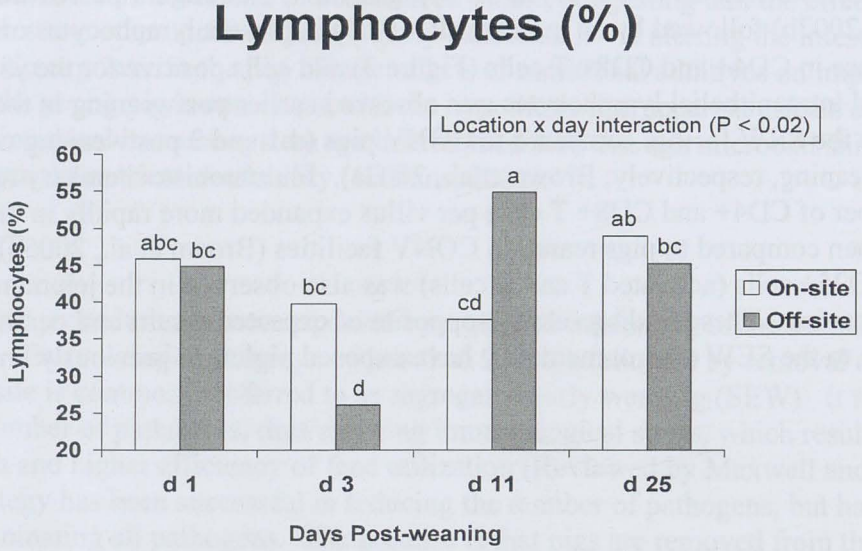


Figure 2. Effect of rearing system on percentage of lymphocytes as a percentage of plasma leukocytes on d1, 3, 11 and 25 postweaning. Pigs were weaned at 19 d of age. Values are means of 4 pigs representing each management system for each day after weaning. [a, b, c] Least squares means with a different superscript differs, $P < 0.03$). Brown et al., 2002b.

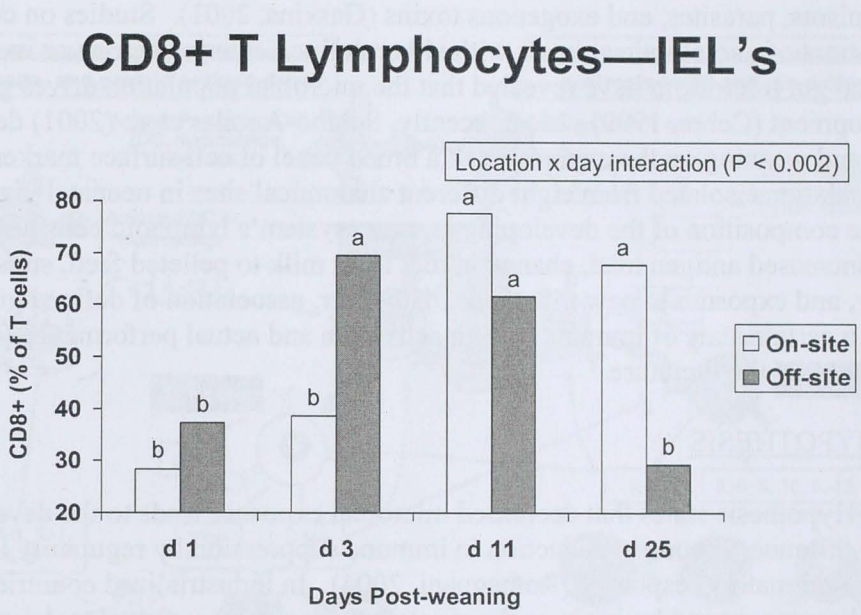


Figure 3. Effect of rearing system on percentage of CD8+ intraepithelial lymphocytes as a percentage of plasma leukocytes on d1, 3, 11 and 25 postweaning. Pigs were weaned at 19 d of age. Values are means of 4 pigs representing each management system for each day after weaning. [a,b,c] Least squares means with a different superscript differs, $P < 0.05$). Brown et al., 2002b.

The increased expansion of cells positive for CD8 represent cytotoxic T cells and/or natural killer cells. The increased expansion of these cells is indicative of a response to eliminate the challenge. SEW pigs had an earlier expansion of CD8+ cells in the jejunum than CONV pigs. This increase was observed on d 1 and 3 in SEW pigs but was not observed until d 11 in CONV pigs, suggesting a faster development of immune function in SEW pigs. Changes observed in T cell subsets in the epithelium and lamina propria of SEW and CONV pigs after weaning may indicate that these immune cells are in an activated state. The greater acquisition of CD4+ cells in the lamina propria of SEW pigs at d 1 after weaning and the different timing of expansion of CD8+ and CD25+ cells over the postweaning period may be attributed to alterations in intestinal bacterial populations in response to the pigs' surrounding environment as reported by King et al. (2005). These data suggest that the differing nursery environment alters intestinal bacterial populations and enteric immune system development/activity which may explain the differences observed in performance.

Although we have sufficient evidence to suggest that early-weaned pigs reared at an off-site facility perform better than pigs reared on-site, information about the mechanisms of this enhanced performance and the relationship between performance and the gut microbiota is lacking. Rearing animals in an off-site facility can reduce the vertical transmission of pathogens from the dam. Immunological stresses will be different in animals reared in the off-site facility as compared to animals reared in the on-site facility, especially at the portal of pathogen entry

such as the gut mucosa. The gastrointestinal tract of animals is the site of complex interactions between the host immune system and various dietary factors, their breakdown products, as well as microorganisms, parasites, and exogenous toxins (Gaskins, 2001). Studies on colonization of the intestinal tract of gnotobiotic animals with either defined enteric bacteria or incompletely defined normal gut microflora have revealed that the microbial population drives gut immune system development (Cebra, 1999). Most recently, Solano-Aguilar et al. (2001) described the effect of age and weaning on the expression of a broad panel of cell surface markers on lymphoid populations isolated from eight different anatomical sites in neonatal pigs. These changes in the composition of the developing immune system's lymphoid cells have been attributed to increased antigen load, change in diet from milk to pelleted feed, stress of moving to a new facility, and exposure to new infections. However, association of defined gut microbial ecology with a certain state of immune system activation and actual performance (health) has not been documented in the literature.

HYGIENE HYPOTHESIS

The Hygiene Hypothesis states that decreased microbial exposure leads to the development of an inappropriate immune response – reduction in immune suppression by regulatory immune cells that control inflammatory responses (Romagnani, 2004). In industrialized countries the incidence of diseases caused by immune dysregulation has risen compared to developing countries and this has been associated with defects in immune regulation (Guarner et al., 2006). A relationship exists between improved hygiene and an increase in allergic disease (Smit et al., 2004). Furthermore, epidemiologic data demonstrate that there has been a steady increase in the incidence of a number of immunoregulatory disorders in westernized countries during the past few decades (Bach, 2002). The hygiene hypothesis attempts to explain that increased incidences of diseases associated with defects in immune regulation are a result of the emphasis on cleanliness within developed societies (urban vs. rural). This concept can be associated with modern swine production when one considers the changes that have been implemented in raising pigs in modern confinement swine facilities compared to outdoor pasture systems that would have historically been the norm. For instance, many technologies geared toward minimizing exposure of pigs to microorganisms, such as all–in-all-out production systems, disinfectant sanitation of facilities, SEW, and antibiotic supplementation can be considered analogous to the same types of technologies (i.e. sanitation, antimicrobial products) that have been implemented in industrialized society. The result is a modern population of pigs or humans that live in a relatively clean environment that are exposed to a very different population of environmental microflora than their historical counterparts.

So the idea of the "hygiene hypothesis" is that decreased microbial exposure early in life leads to the development of an inappropriate immune response, specifically, as suggested by recent evidence, a reduction in immune suppression by regulatory immune cells that control inflammatory responses. Immune activation and suppression has been closely linked to dendritic cells or other antigen presenting cells which acquire signals from the microenvironment and convey these to naïve T cells, instructing their development into polarized T_H1, T_H2, or regulatory T cells (McGuirk et al., 2002; Hansen et al., 1999). Although neonates at birth are skewed toward T_H2 T cell immune responsiveness, early exposure to inflammatory challenges from the microbiota skew T cells toward a T_H1 polarization (McGuirk et al., 2002). It is possible

that induction of inflammatory responses from high bacterial turnover is instrumental to the development of the regulatory network as depicted in Figure 4.

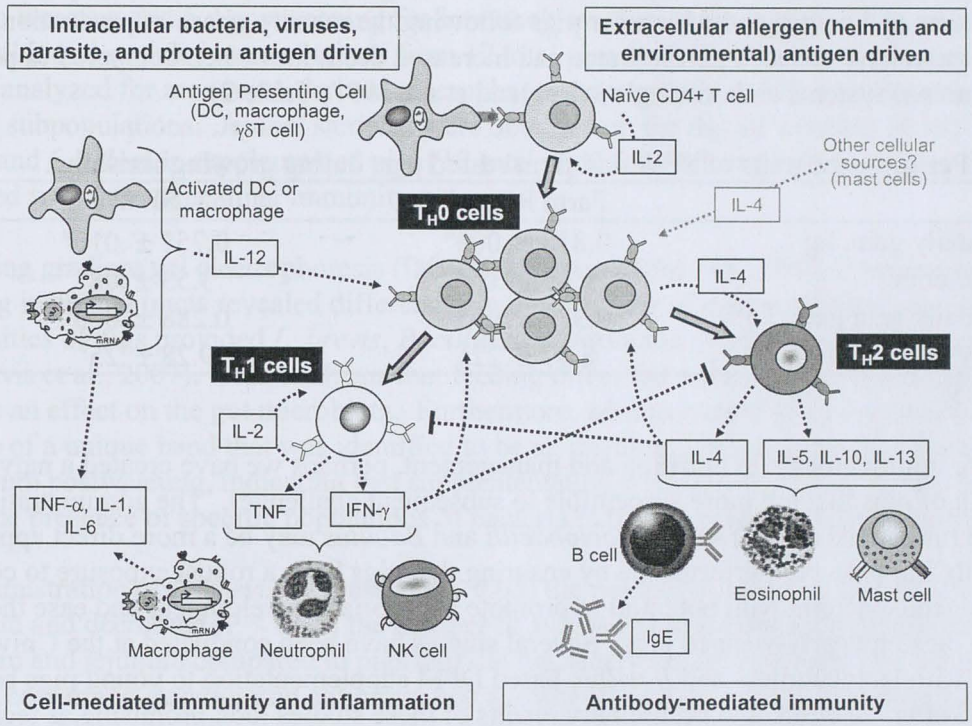


Figure 4. Schematic illustrating bacterial signals guiding the development of naïve T helper cells (CD4+) toward T_H1 or T_H2 polarization: Intracellular bacterial antigen presented by antigen presenting cells and the presence of IL-12 skew T cell development toward an inflammatory $T_H1$immune response, whereas extracellular antigenic material skew T cell development toward a T_H2 immune response.

The model also raises the question whether certain bacteria carry signature molecules that are better suited to induce activation of regulatory cells and to protect against atrophy than other bacteria and predicts that frequent antigenic challenge from an array of bacterial and viral challenges may be needed for a balanced development of the immune system and the prevention of inflammatory diseases (Matricardi and Bonini, 2000; Jeannin et al., 1998). However, instead of a T_H1 versus T_H2 paradigm, it may be the pro- versus anti-inflammatory axis with a robust regulatory T cell network that has to be considered central to the balance and to the prevention of either T_H1 and/or T_H2 imbalance (Yazdanbakhsh et al., 2001).

In relation to animal production, dysregulation or imbalance in the inflammatory vs. anti-inflammatory pathways may result in growth depression from over-exuberant inflammation responses, or disease susceptibility from lack of inflammation when needed to combat pathogens. Many of the techniques utilized in swine management to improve performance do so by limiting the potential exposure of pigs to inflammatory stimuli. Our understanding of how one improves pig performance by dietary manipulation and/or rearing in an isolated environment (SEW) has resulted in improved pig performance as discussed earlier as long as the pig remains in isolation. Unfortunately, both the dietary approach and the SEW approach lead to a

population of naïve pigs that can become hyper-responders to immune challenges encountered later in life. Dietary plasma protein, as indicated previously, is a good example of how nutrition can produce increased immune responsiveness following its removal. Similarly, pigs reared in SEW systems and comingled with other pigs following the nursery phase of production have been shown to have reduced performance and increased death loss when compared to pigs reared in conventional systems and comingled (Ragland et al., 1997; Table 2).

Table 2. Performance traits of SEW and farm reared pigs during growing/finishing.

	Farm Reared	SEW
Average daily gain, kg	$0.818 \pm .009^{a}$	$0.751 \pm .010^{b}$
Feed Efficiency	$3.31 \pm .07^{a}$	$3.53 \pm .08^{b}$
Average daily lean gain, kg	$0.320 \pm .005^{a}$	$0.288 \pm .006^{b}$
Efficiency of lean gain	$8.47 \pm .22^{a}$	$9.28 \pm .25^{b}$

^{a,b} $P < 0.05$

With these improvements in nutrition and management, perhaps we have created a naïve population of pigs that are more susceptible to subsequent challenges. The administration of direct-fed microbials (DFM) such as *lactobacilli* and *Bacillus* may be a more direct approach to beneficially improve pig performance by ensuring that pigs have a robust exposure to common non-pathogenic bacteria with potential to promote appropriate development and ease the transition faced by early-weaned pigs. Several studies have been conducted at the University of Arkansas with lactobacillus- and *Bacillus*-based DFM supplementation to young pigs both before and after weaning, and preliminary results of these studies look promising as techniques to enhance appropriate immune development and performance in the young pig.

DIRECT-FED MICROBIAL SUPPLEMENTATION

Research conducted at the University of Arkansas has investigated the effects of two direct-fed microbials for young pigs: 1) *Lactobacillus brevis* administered to piglets via milk supplementation during the lactation phase of production and via water during the nursery period, and 2) two strains of *Bacillus subtilis* administered through the feed in the nursery period. The following studies were conducted to evaluate the effects of these direct-fed microbials and antibiotic supplementation on growth performance, the gastrointestinal microbial community, maturation of goblet cells, and immune development of young pigs (Maxwell et al., 2005).

During lactation, piglets were administered milk supplement with and without *L. brevis* via a liquid feeding system in addition to sow's milk. The supplemented milk containing *L. brevis* and devoid of antibiotics was administered to half of the litters within the farrowing group, whereas the remaining litters were administered milk devoid of *L. brevis* and antibiotics. Following the lactation phase, pigs that were administered milk supplement with and without *L. brevis* prior to weaning were maintained on their respective treatment through the nursery period by administering *L. brevis* through the watering system. Pigs within the two *L. brevis* treatments were then allotted to one of three nursery diets. The three dietary treatments fed during the nursery included: 1) Control 2) *Bacillus*, and 3) antibiotic (Carbadox). Pig body weight was measured at the end of each nursery phase. On d 10, 20, and 38 after weaning, a total of 12 pigs (two from each experimental treatment) were euthanized for the collection of gastrointestinal

samples (duodenum, jejunum, and ileum) to determine microbial populations and goblet cell enumeration. To evaluate the effects of *L. brevis* on intestinal immune development, jejunal tissues from an additional 4 pigs from only the Control and *L. brevis* treatments were obtained 5 d prior to weaning and 5 d postweaning for immunohistochemistry evaluation. In addition, peripheral blood samples were obtained from 12 total pigs on d 10, 20, and 38 postweaning and samples analyzed for monocyte-derived macrophage phagocytosis and flow cytometric analysis of T cell subpopulations. Jejunal samples were obtained on the day of weaning (d 19) from 6 Control and 6 *L. brevis*-supplemented pigs (12 total pigs) for evaluation of expression of genes associated with gastrointestinal immunity.

Denaturing gradient gel electrophoresis (DGGE) analysis of the 16S rDNA from the microflora of the pig intestinal tracts revealed differences in the diversity of gastrointestinal microbial communities of pigs provided *L. brevis*, *Bacillus*, and antibiotic compared to unsupplemented pigs (Davis et al., 2007). This confirms that feeding direct fed microbials as well as antibiotics can have an effect on the gut microbiota. Furthermore, administration of *L. brevis* resulted in the presence of a unique band that was identified to be an unculturable, gram positive bacterium in the jejunum postweaning, indicating that supplementation with specific direct-fed microbials can induce the presence of specific populations of bacteria in the gastrointestinal microenvironment.

The administration of *L. brevis* increased ($P < 0.05$) the number of acidic goblet cells in the duodenum and decreased ($P < 0.05$) the number of sulfated mucin-producing goblet cells in the duodenum and jejunum compared to pigs that did not receive *L. brevis* (Table 3). Likewise, pigs fed diets containing *Bacillus* and antibiotic had a lower ($P < 0.05$) number of sulfated mucin-producing goblet cells in the duodenum and jejunum than pigs fed the control diet during the nursery period. Differentiation of mucin-secreting goblet cells is influenced by the gastrointestinal microbial flora (Sharma et al., 1995), and the changes in the microflora observed in this study from DFM and antibiotic supplementation resulted in alterations in goblet cell maturation. Goblet cells differentiate as they mature, and the chemical composition of the mucin within the goblet cell changes (Dunsford et al., 1991). Immature goblet cells produce neutral mucins containing little sialic acid and as they mature, the mucins become increasingly sialated, or acidic (Specian and Oliver, 1991). Acidic goblet cells can also be sulfated to form two distinct acidic subtypes, including the sialomucin and sulfomucin groups. Sulfated mucins seem to be less susceptible to bacterial degradation and may have a more predominate function in the absence of an appropriately developed immune system (Deplancke and Gaskins, 2001). In the present study, the administration of *L. brevis*, *Bacillus*, and antibiotic decreased the number of sulfuric goblet cells in the duodenum and jejunum, suggesting a maturity in the development of mucus-secreting goblet cells when pigs were provided DFM or antibiotic supplementation.

Table 3. Effect of *L. brevis* and dietary treatments during the nursery period on neutral, acidic, and sulfuric goblet cell enumeration (average number of goblet cells per villus) in the duodenum and jejunum of the pig small intestine.

		Lactobacillus brevis[1]			Nursery Diet[2]			
Tissue	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	18.52^{a}	10.88^{b}	0.71	17.76^{a}	12.57^{b}	13.77^{b}	0.88

[1] Data are the least squares means of 18 pigs (6 pigs/treatment/sampling day) sampled over 3 days (d 10, 20, and 38) during the postweaning period.
[2] Data are the least squares means of 12 pigs (4 pigs/dietary treatment/sampling day) sampled over 3 days (d 10, 20, and 38) during the postweaning period.
[a,b] Means in a row within a treatment without a common superscript are different ($P < 0.05$).

Pigs fed antibiotics in the absence of *L. brevis* supplementation had a lower percentage of phagocytic macrophages compared to pigs fed the Control diet or Bacillus (Figure 5). Macrophages from pigs fed *Bacillus* phagocytosed a greater number of sheep red blood cells than pigs fed antibiotics (Figure 6).

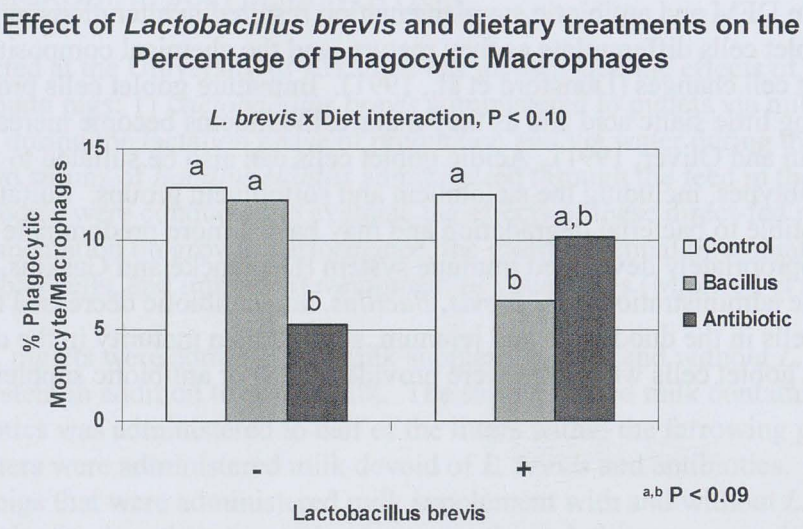


Figure 5. Effect of *Lactobacillus brevis* administration and dietary treatments fed during the nursery period on the percentage of phagocytic monocyte-derived macrophages isolated from the peripheral blood of pigs during the postweaning period (*L. brevis* x diet interaction, $P < 0.10$; [a, b] $P < 0.09$). Maxwell et al., 2005.

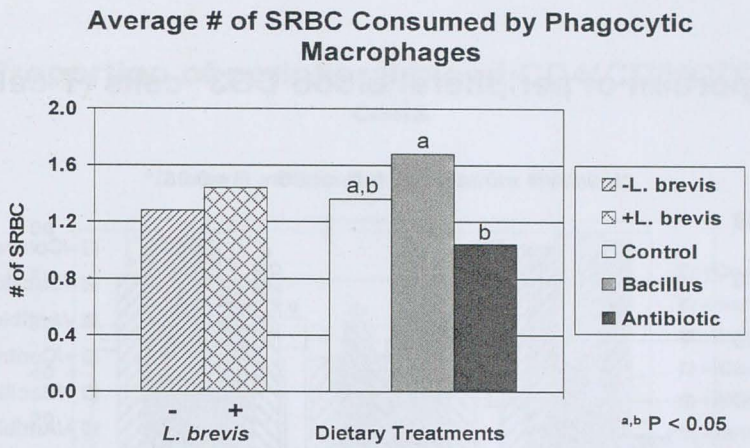


Figure 6. Main effects of *Lactobacillus brevis* administration and dietary treatments during the nursery period on the average number of sheep red blood cells consumed by phagocytic monocyte-derived macrophages isolated from the peripheral blood of pigs postweaning. Values are means of 6 pigs/treatment for *L. brevis* and 4 pigs/treatment for nursery diets. Least squares means with a different superscript differ, $P < 0.05$). Maxwell et al., 2005.

Although both *Bacillus* and antibiotic supplementation decreased the percentage of phagocytic macrophages compared to Controls, the macrophages from *Bacillus*-fed pigs that were phagocytic had a greater functional capacity to phagocytose sheep red blood cells than pigs fed antibiotics. This provides an illustration of how antibiotics decrease the inflammatory phagocytic response of the innate immune system and also inhibits the functional capacity of individual phagocytic macrophages to engulf foreign material. Whereas supplementation with the DFM fails to induce a similar anti-inflammatory effect as antibiotic supplementation, the administration of *Bacillus* organisms to the young pig does not result in the same inhibition of the macrophage's ability to phagocytose.

On d 20 after weaning, pigs fed the antibiotic diet in the absence of *L. brevis* and pigs fed the Control diet and provided with *L. brevis* had a greater proportion of T cells as indicated by the CD3+ marker (Figure 7). However on d 38 after weaning, *Bacillus*-fed pigs not provided with *L. brevis* and pigs receiving *L. brevis* supplementation had the greater CD3+ population. Antibiotic and *L. brevis* decreased T cell proportions from d 20 to 38 while pigs fed *Bacillus* diets (both) increased T cell proportions over same time period. These data indicate that the development of T cell immunity is altered in response to these supplements and this response is very interactive and complex. The same pattern observed for the CD3+ T cell population is repeated with the CD3+MHCII+ T cells, indicating this CD3+ population was an experienced subset of T cells (Figure 8). Figure 9 shows the activated proportion (CD25+) of the double positive T cell population (CD4+CD8+). All treatments including both direct-fed microbials and the antibiotic had a higher proportion of activated CD4+CD8+ cells than pigs fed the Control diet without *L. brevis* on d 10 after weaning. Interestingly, the activated population decreased in these treatments on d 20 after weaning while the Control pigs without *L. brevis* did not change, with all treatments increasing at d 38 after weaning, illustrating direct fed microbials and antibiotics may help the pig avoid the strong inflammatory effects often observed at the stress of weaning.

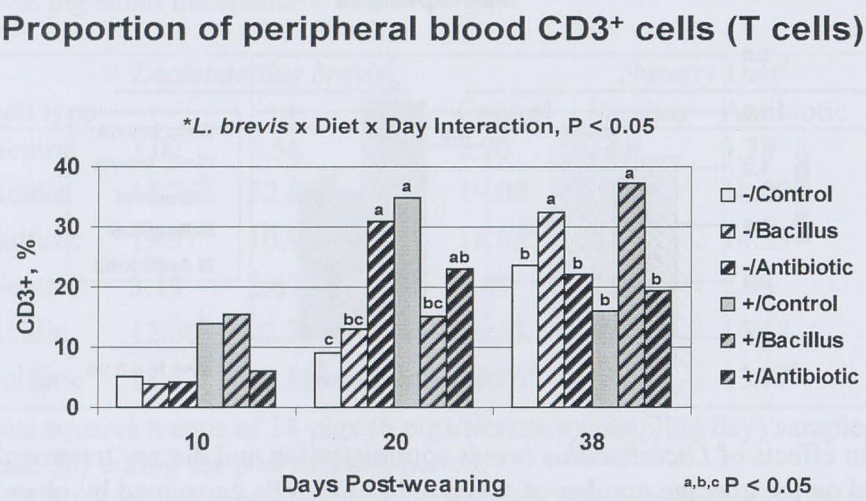


Figure 7. Effect of *Lactobacillus brevis* (- or +) and dietary supplementation with *Bacillus* and antibiotic on the proportion of $CD3^+$ lymphocytes (T cells) within the peripheral blood mononuclear cell population d 10, 20, and 38 postweaning (1E1 x diet x day interaction, P = 0.03); [a, b, c] Within each day postweaning, means without a common letter differ, P < 0.05). Maxwell et al., 2005.

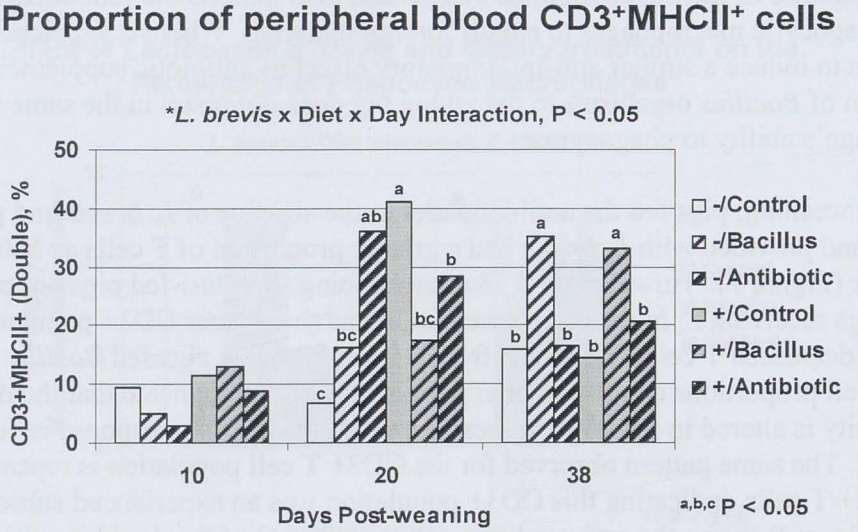


Figure 8. Effect of *Lactobacillus brevis* (- or +) and dietary supplementation with *Bacillus* and antibiotic on the proportion of $CD3^+MHCII^+$ lymphocytes within the peripheral blood mononuclear cell population d 10, 20, and 38 postweaning (*L. brevis* x diet x day interaction, P = 0.04; [a, b, c] Within each day postweaning, means without a common letter differ, P < 0.05). Maxwell et al., 2005.

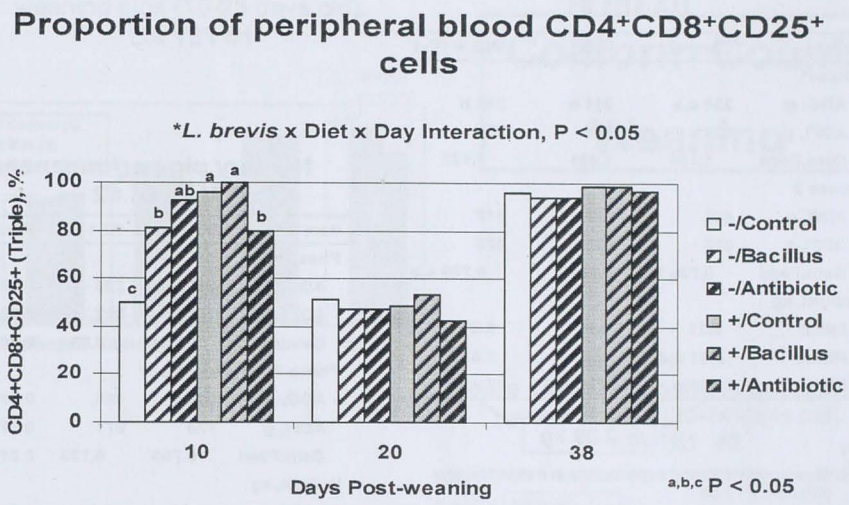


Figure 9. Effect of *Lactobacillus brevis* (- or +) and dietary supplementation with *Bacillus* and antibiotic on the proportion of $CD25^+$ lymphocytes within the $CD4^+CD8^+$ population isolated from peripheral blood on d 10, 20, and 38 postweaning (*L. brevis* x diet x day interaction, P = 0.004; [a, b, c] Within each day postweaning, means without a common letter differ, P < 0.05). Maxwell et al., 2005.

To further support the anti-inflammatory effects of *L. brevis,* immunohistological evaluation revealed a lower (P < 0.05) number of T helper lymphocytes (CD4+, 11.7 vs. 7.9) on jejunal villi compared to unsupplemented pigs, illustrating that supplementation with *L. brevis* resulted in less infiltration of T helper lymphocytes within the jejunum compared to unsupplemented pigs. Less lymphocyte infiltration in the gastrointestinal tract is indicative of less inflammation, and suggests *L. brevis* may promote a balanced, regulatory immune response in the young pig. Regulation of inflammatory responses may be one mechanism by which *L. brevis* supplementation improves nursery pig performance.

Interestingly, the induction of a growth performance response postweaning from the administration of *L. brevis* pre-weaning seems to be dependent upon an inflammatory challenge during the time of *L. brevis* supplementation. Not all experiments with *L. brevis* have resulted in increased subsequent performance in nursery pigs postweaning. In two experiments conducted, we observed an increase in pig weight at the end of the nursery phase in experiment 1, whereas in experiment 2, pig weight at the end of the nursery period was similar between pigs offered *L. brevis* during lactation compared to Control pigs (Figure 10). It is interesting to note that jejunal coliform counts tended to be reduced in the pre-weaning period (d 7 to 12 post-farrowing) in pigs fed *L. brevis* in both experiments; however, coliform levels were approximately 2 to 3 logs lower during the pre-weaning period in all sections of the GI tract evaluated in experiment 2 compared to experiment 1 (Figure 11). A similar pattern was observed at weaning (Figure 12). This suggests that one might expect to find improved performance in pigs where a sufficient challenge threshold has been reached but not see a response in less challenged pigs, providing insight into the importance of an inflammatory stimulus to induce regulatory immune control by *L. brevis*.

Nursery pig performance.
UA 1E1 #1

Item	Control	Milk	Milk + 1E-1
Phase1			
ADG, g	239 a,b	211 b	258 b
ADFI, g	228	211	250
Gain:Feed	1.109	1.031	1.139
Phase 2			
ADG, g	466	487	517
ADFI, g	633	620	673
Gain:Feed	0.736 b	0.790 a	0.769 a,b
Weight, kg			
Initial	5.21	5.50	6.31
Phase 1	8.51 c,d	8.26 d	9.88 c
Phase 2	15.03 e	15.19 e,f	17.12 f

+ 2.09 kg

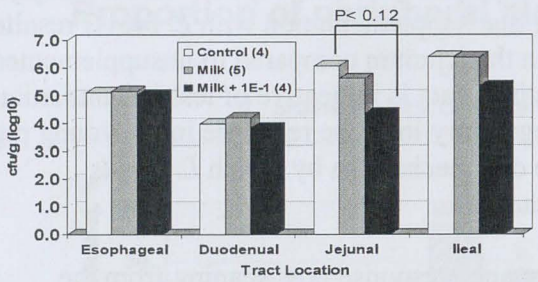
a,b Means with differing superscripts are significantly different; P<0.05
c,d Means with differing superscripts are significantly different; P=0.07
e,f Means with differing superscripts are significantly different; P=0.11

Nursery pig performance.
UA 1E1 #2

Item	Milk	1E-1	P-value
Phase1			
ADG, g	238	225	0.62
ADFI, g	295	322	0.43
Gain:Feed	0.784	0.704	0.06
Phase 2			
ADG, g	508	485	0.48
ADFI, g	724	671	0.17
Gain:Feed	0.703	0.723	0.51
Weight, kg			
Initial	6.63	6.40	0.75
Phase 1	9.91	9.43	0.60
Phase 2	17.02	16.20	0.53

Figure 10. Summary of growth performance in two *Lactobacillus brevis* studies conducted at the University of Arkansas. Unpublished data.

Figure 11A. Mean coliform populations for preweaning pigs (9-13 days old).
UA 1E1 #1



Coliform Counts
Pre-Weaning

Figure 11B. Mean coliform populations for preweaning pigs (7-11 days old).
UA 1E1 #2

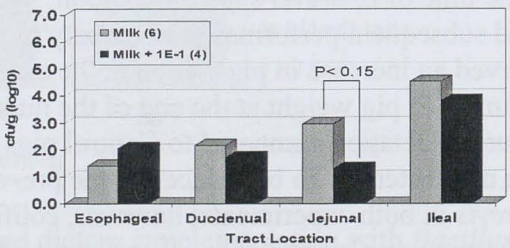
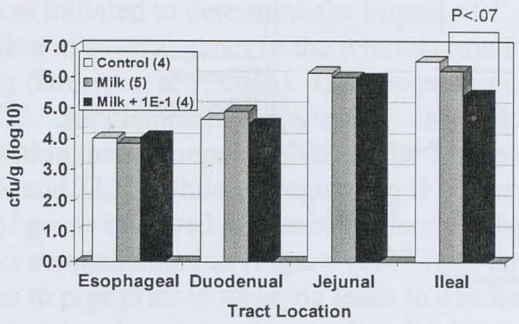


Figure 11. Effect of milk replacer supplementation with *Lactobacillus brevis* during lactation on pre-weaning (approximately 10 d of age) coliform counts in two trials conducted at the University of Arkansas. Unpublished data.

Figure 12A. Mean coliform populations for weaning pigs (19-23 days old).
UA 1E1 #1



Coliform Counts

Weaning

Figure 12B. Mean coliform populations for weaning pigs (20-24 days old).
UA 1E1 **#2**

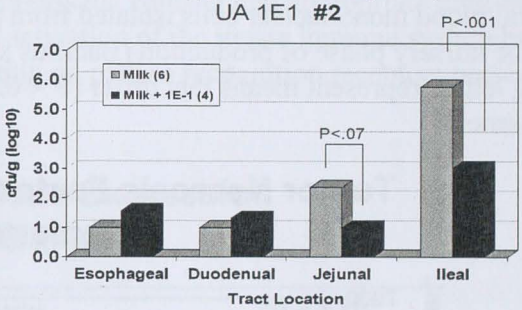


Figure 12. Effect of milk replacer supplementation with *Lactobacillus brevis* during lactation on coliform counts at weaning (approximately 21 d of age) in two trials conducted at the University of Arkansas. Unpublished data.

An experiment evaluating the cytokine profiles induced by supplementation with two strains of *Bacillus subtilis* to nursery pigs further corroborates that an inflammatory response is a requirement for the induction of regulatory immune control (unpublished data). Pigs fed *Bacillus* cultures had a higher ($P < 0.05$) production of IL-1β from LPS stimulated peripheral blood mononuclear cells (PBMC) on d 20 after weaning when compared to d 42 after weaning, while IL-1β production was similar ($P > 0.10$) on d 20 and 42 after weaning in pigs fed diets devoid of *Bacillus* cultures (*Bacillus* × day interaction, $P = 0.05$; Figure 13). The production of TNF-α from LPS stimulated PBMC were similar ($P > 0.10$) among pigs fed diets with or without *Bacillus* cultures on d 20 after weaning but by d 42 after weaning pigs fed diets containing *Bacillus* cultures tended to have a lower ($P < 0.10$) production of TNF-α from LPS stimulated PBMC compared to pigs fed diets devoid of *Bacillus* cultures (*Bacillus* × day interaction, $P = 0.11$; Figure14). The induction of inflammatory TNF-α and IL-1β, by *Bacillus*, followed by the subsequent decrease in these levels later in the nursery period compared to unsupplemented pigs, suggests a regulatory immune induction in response to the initial inflammation.

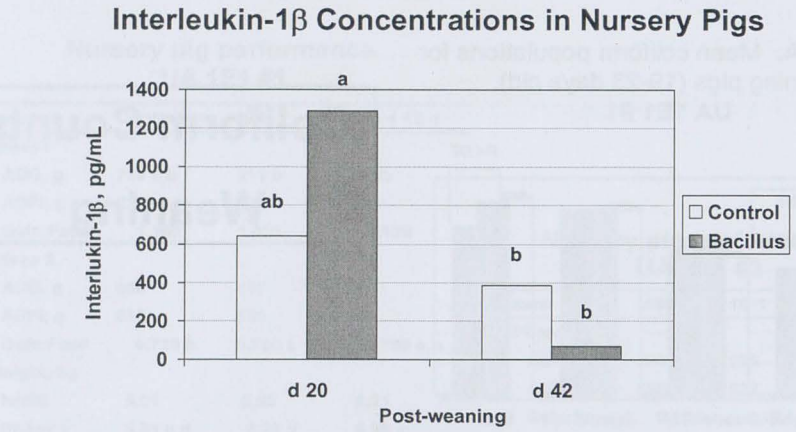


Figure 13. Monocyte IL-1 β elaboration responses from LPS stimulated cell cultures of peripheral blood mononuclear cells isolated from pigs fed diets with or without *Bacillus* cultures during the nursery phase of production (Bacillus x day interaction, P = 0.05). [a, b] Bars with differing letters represent means that differ (P < 0.05). Values represent the mean of 16 pigs/treatment.

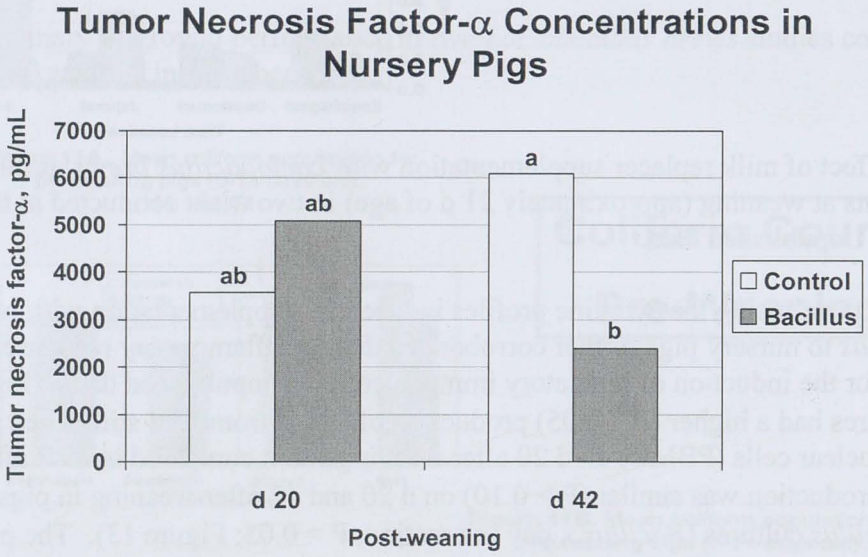


Figure 14. Monocyte TNF-α elaboration responses from LPS stimulated cell cultures of peripheral blood mononuclear cells isolated from pigs fed diets with or without *Bacillus* cultures during the nursery phase of production (Bacillus x day interaction, P = 0.11). [a, b] Bars with differing letters represent means that differ (P < 0.10). Values represent the means of 16 pigs/treatment.

LACTOBACILLUS BREVIS EFFECTS ON TOLL-LIKE RECEPTOR SIGNALING

Several studies in our laboratory have reported that adding *L. brevis* to the supplemental milk replacer during lactation in a conventional facility can improve the young pigs' growth performance (Brown et al., 2003; Davis et al., 2003) and villus/crypt architecture (Brown et al.,

2003) after the weaning process. Furthermore, supplementing pigs with *L. brevis* in the milk replacer during lactation in a conventional facility has been shown to reduce the number of T cells within the jejunal section of the intestinal tract of the young pig after weaning, which could lead to decreased activation of the gut immune system (Halbrook et al., 2005a). Therefore, a study was initiated to determine the impact of *L. brevis* fed in a milk replacer during lactation on expression of several genes in the jejunum involved in the Toll-like receptor (TLR) pathway at weaning (Brown et al., 2006a). Gene expression levels were determined using quantitative real-time PCR. Supplementing pigs with *L. brevis* during the lactation period down-regulated several genes within the jejunum involved in the TLR pathway, specifically SARM, TIRP, TLR4, TRAF6, and TLR9 while up-regulating IFN-γ at weaning (Brown et al, 2006a). Gene expression levels of genes involved in mucin production, T_H2 response, and tolerance were not affected by *L. brevis* supplementation (Figure 15). These data support the concept that supplementation of *L. brevis* to pigs prior to weaning leads to a reduced inflammatory response and suggests that the mechanism involves several genes involved in the Toll-like receptor pathway within the jejunum. Involvement of the TLR pathway supports the concept that appropriate immune development in the neonate is dependent upon activation of the innate immune system by components of the microbiota environment acting on pattern recognition receptors present on cells of the gastrointestinal tract.

Changes in Gene Expression
d0 (Weaning)

Control vs. L. brevis				*P < 0.10
INF-γ*	++	MUC3A	=	
CD4	=	MyD88	--	Fold Change
CD69	--	IRAK4	--	-- -1 to -2
CD16	--	TRAF6*	--	- -1 to 0
IL-10	++	TIRP*	--	= 0 to 1
TLR-4*	--	SARM*	--	+ 1 to 2
TLR-9*	--	NFkB1	--	++ 2 to 3

Figure 15. Changes in gene expression in the jejunum at weaning in pigs fed milk replacer containing *Lactobacillus brevis* or the milk replacer devoid of *L. brevis*. Genes included in the Real Time-PCR assay included (Gene symbol): MUC3A, CD4, CD16, CD 69, TIRP, TLR4, TLR9, TRAF6, IRAK4, INF-γ, IL10, MYD88, NFKB1, SARM1; Means are for 6 pigs/treatment. Brown et al., 2006a.

YEAST CELL WALL POTENTIAL

Polysaccharides from the yeast cell wall have been shown to interact with the immune system of the host by binding to specific receptors on monocytes/macrophages associated with the Toll-Like receptor pathway and eliciting an immune response (Kogan and Kocher, 2007). The effects of a yeast cell wall product on pig performance and immunocompetence was evaluated in five nursery pig trials conducted at the University of Arkansas. A total of 412 pigs were involved in the studies evaluating three phase nursery studies (Maxwell, 2004). During Phase 1, yeast cell wall supplementation improved ($P < 0.02$) feed efficiency compared to pigs fed the basal diet, whereas improvement in average daily gain approached significance (Table 4, $P = 0.11$). During the first week of Phase 3, average daily gain ($P < 0.05$) and feed efficiency ($P < 0.05$) were improved in pigs fed yeast cell wall when compared to pigs fed the basal diet. The fifth study evaluated the impact of yeast cell wall on performance and some immune function parameters (Davis et al., 2004). In this study, yeast cell wall-supplemented pigs had an increase in average daily gain and efficiency of gain ($P < 0.05$) and an increase ($P < 0.05$) in the phagocytic capacity of jejunal lamina propria macrophages compared to pigs fed the basal diet (Table 5).

Table 4. Summary of the effect of yeast cell wall on growth performance in 5 trials.

	Yeast Cell Wall			
	0%	0.2%	0.3%	P-value
ADG, g				
Phase 1[c]	147 + 9	168 + 16	166 + 10	0.26
Phase 2	372 + 12	369 + 22	391 + 14	0.56
Phase 3a[d]	461 + 14	516 + 23	501 + 18	0.07
Feed:gain				
Phase 1[d]	1.709 + .067[a]	1.388 + .126[b]	1.493 + .080[b]	0.02
Phase 2	1.437 + .085	1.428 + .160	1.195 + .104	0.20
Phase 1-2[f]	1.379 + .025[a]	1.375 + .046[ab]	1.273 + .030[b]	0.03
Phase 3a[d]	1.700 + .028[a]	1.586 + .046[b]	1.610 + .036[ab]	0.03

[a,b] Means in a row with no letters in common differ ($P < 0.05$).
[c] Contrast: 0 vs. 0.2 + 0.3% Yeast cell wall; $P = 0.11$.
[d] Contrast: 0 vs. 0.2 + 0.3% Yeast cell wall; $P < 0.05$.
[e] Contrast: 0 vs. 0.2 + 0.3% Yeast cell wall; $P < 0.10$.
[f] Contrast: 0.2% vs. 0.3% Yeast cell wall; $P < 0.10$.

Table 5. Macrophage phagocytosis response of weanling pigs fed phosphorylated mannans or a basal (control) diet.

	Control	Mannan	SEM	$P =$
Monocyte/macrophage phagocytosis				
Blood[a]				
% Phagocytic	15.7	16.5	1.73	0.769
Average SRBC	1.62	1.70	0.07	0.432
Lamina propria[b]				
% Phagocytic	24.2	26.7	1.94	0.366
Average SRBC	2.31	2.63	0.11	0.051

[a]Peripheral blood samples were obtained from 36 pigs on d 14 after weaning to determine the percentage of phagocytic monocyte/macrophages and average number of sheep red blood cells (SRBC) phagocytosed by monocyte/macrophages isolated from blood. Values are means of eight pens representing each dietary treatment.

[b]Jejunal samples were obtained on d 19, 21, 24, and 26 from eight pigs on each day for the isolation of lamina propria macrophages. Values are means of four pens representing each dietary treatment.

Although the function of lymphocytes derived from peripheral blood was not impacted by yeast cell wall supplementation, yeast cell wall did enhance innate immune function in the gastrointestinal system which may explain the improved performance observed in yeast cell wall fed pigs and represents another example of enhanced performance associated with early TLR signaling.

SUMMARY

Studies in the early-weaned pig indicate that the mechanism whereby nutrition and/or management systems have improved growth performance is likely through a reduction of the negative impacts of the intestinal microbiota. These management systems have enhanced performance but may have resulted in stunting the development of an appropriate and functional immune system. Research at the University of Arkansas involving the feeding of direct fed microbials to the piglet during lactation in a milk replacer and/or during the nursery phase improved the growth performance of pigs during the nursery period, and resulted in potentially beneficial alterations in gastrointestinal microflora, decreased the percentage of phagocytic macrophages while enhancing macrophage function. Benefits of supplementation were also observed in reduced gene expression of genes involved in the TLR pathway, enhanced goblet cells maturation, increased activated and γδT cell subsets, and altered immune cell populations in a manner consistent with a reduced inflammatory response. This supports the concept that early exposure to selected microbials impacts the innate and adaptive immune function in piglets and may reduce susceptibility to postweaning challenges. Although many questions remain, these finding suggest early signaling through the innate immune system may play a pivotal role in appropriate immune development that translates into enhanced growth in the young pig.

TAKE-HOME MESSAGE

The advent of early weaning in the swine industry for efficient and economic pig production has resulted in pigs with a naïve, underdeveloped immune system at the time of weaning when the intestinal microflora can be adversely impacted resulting in higher numbers of potentially pathogenic coliforms and a decline of favorable lactobacilli. As a result, pigs are ill equipped to deal with pathogenic challenges encountered when passive immunity acquired from the sow is no longer available. Several examples of improved postweaning performance in the young pig suggest that much of the improvement observed with enhanced nutritional regimes for the early-weaned pig (e.g. plasma protein) and management systems such as segregated early weaning (SEW) may be through decreasing the effects of rapid changes in intestinal microbiota on pig health and performance. Both plasma protein and rearing pigs under SEW management conditions are analogous to rearing a pig in isolation affording some protection from health challenges and perhaps an unintended consequence is stunting development of an appropriate and functional immune system. The concept that decreased microbial exposure early in life leads to the development of inappropriate immune responses is termed the "hygiene hypothesis" and is believed to explain the increased prevalence of allergy and asthma in children raised in developed societies. Recent evidence suggests that these inappropriate immune responses result from the dysregulation of immune suppression by regulatory immune cells that control inflammatory responses. Although a relatively stable microbiota eventually establishes in the mature pigs' digestive tract, it requires a considerable amount of adaptation during the early life periods. The development of direct-fed microbials (DFM) and/or signatory molecules isolated from select bacteria and/or yeast may have merit by simulating the environment in the gastrointestinal tract of the young pig to promote appropriate immune development and ease the postweaning transition faced by the early-weaned pig. Research at the University of Arkansas that involved feeding DFM to piglets during lactation in a milk replacer and/or during the nursery phase of production has improved growth performance and resulted in goblet cell maturation, decreased the percentage of phagocytic macrophages while enhancing macrophage function, decreased gene expression involved in the Toll-like receptor pathways, and increased activated- and γδ-T cell subsets. Similarly, feeding yeast cell wall components to nursery pigs or to sows during lactation has resulted in improved pig nursery growth performance and enhanced the immune system. Although many questions remain, these finding suggest early signaling through the innate immune system may play a pivotal role in the development of appropriate immune responses that translates into enhanced growth in the young pig.

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