

## Original Article

# Benefits of pre-, pro- and Syn-biotics for lung angiogenesis in malnutritional rats exposed to intermittent hypoxia

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**Abstract:** Extremely low birth weight and reduced caloric intake have significant adverse effects on lung development and are risk factors for bronchopulmonary dysplasia. Vascular endothelial growth factor (VEGF) is highly involved in lung microvascular development, and may be affected by nutritional status. To test the hypothesis that suboptimal nutrition decreases VEGF signaling in formula-fed neonatal rats, and to determine whether supplementation with probiotics, prebiotics, or synbiotics ameliorate the effects, rat pups at birth (P0) were placed in room air (RA) or intermittent hypoxia (12%) during hyperoxia (50% O<sub>2</sub>) from birth to P3. The pups were either maternally-fed; or formula-fed with or without supplementation. Formula-fed pups were separated from their mothers at birth and hand-gavaged every 3 hours. Lung VEGF signaling was determined on P3. In RA, all formula-fed groups were significantly growth suppressed with decreased lung weights. Hyperoxia had a less remarkable effect on body weight; and mean lung weight was lower only in the unsupplemented formula-fed group. Lung VEGF was decreased in all formula-fed RA and hyperoxia groups, except the probiotics group. In RA, sVEGFR-1 levels were elevated in all formula-fed groups except the synbiotics group. However in hyperoxia, sVEGFR-1 levels were higher in the unsupplemented formula group. All genes involved in angiogenesis were downregulated in the formula-fed groups compared to maternally-fed. Formula feeding results in significant malnutrition associated with decreased lung size and lung VEGF levels in neonatal rat pups. Probiotic supplementation prevented the adverse effects of combined hyperoxia and suboptimal nutrition on lung VEGF suggesting preservation of angiogenesis.

**Keywords:** Corticosterone, lungs, malnutrition, stress, VEGF, sVEGFR-1

## Introduction

Extremely low birth weight (ELBW) preterm infants are exposed to numerous stressors including compromised nutritional status during the early postnatal period. Suboptimal nutrition has significant effects on lung development, structure and size [1]. The relationship between nutrition and lung development is particularly significant in ELBW preterm infants with increased energy expenditure and decreased caloric intake due to poor suckling and fluid restriction [2]. Studies have demonstrated a relationship between poor nutrition and the development of bronchopulmonary dysplasia

(BPD), a form of chronic lung disease (CLD), in preterm infants who require prolonged supplemental oxygen therapy [3-6]. Further animal studies show a harmful relationship between malnutrition and hyperoxia resulting in reduced lung growth and DNA content [7], decreased alveolar growth [8], and low alveolar number and collagen deposition [9]. Malnutrition alone decreased alveolar fluid clearance by 38% [10] and 35% loss of alveoli within 72 hours of calorie restriction, an effect that was reversed with refeeding [11, 12]. These studies provide strong evidence that undernutrition is a key factor in pathologic lung development [13]. The mechanism(s) underlying the effects of malnutri-

tion on hyperoxia-induced lung damage may involve disturbances in lung angiogenic factors.

Vascular endothelial growth factor (VEGF) is a potent mitogen and inducer of endothelial cell growth and proliferation [14]. It is high in breast milk and is important for normal growth and development of the offspring [15-17]. It is highly expressed in epithelial cells during differentiation of human fetal lungs in vitro and plays a key role in regulation of lung microvascular maturation [18]. Exposure to high concentrations of oxygen during lung development results in decreased lung VEGF protein and mRNA [19]. Recovery from hyperoxia increases VEGF expression in alveolar epithelial cells suggesting a role for VEGF in microvascular repair [20]. VEGF exerts its biological effects via two tyrosine kinase receptors, VEGFR-1 and VEGFR-2. Both receptors have an extracellular ligand-binding domain, a transmembrane domain, a tyrosine kinase domain; and a downstream carboxyl terminal region [21]. Soluble VEGFR-1 (sVEGFR-1) is a truncated, splice variant of the membrane type and has only the extracellular domain. It functions as endogenous dominant negative regulators of VEGF by complexing with VEGF with similar affinity as the membrane receptor, thereby preventing downstream signal transduction [22]. Previous studies demonstrated that sVEGFR-1 is abundant in baboon lung homogenates [23] and elevated at birth and during the first few weeks of life in tracheal aspirates of preterm infants developing BPD [24].

There has been growing evidence to support a role for commensal microbes on the human intestinal development and animal health. Studies suggest that the physiological benefits can accrue at sites distant from where prebiotics and probiotics are administered. This may be due to the positive impact of probiotics on the immune system. In the present study, we sought to evaluate the hypothesis that supplementation with probiotics, prebiotics or synbiotics during sub-optimal nutrition has positive effects on lung angiogenesis. Because a significant number of ELBW infants are also exposed to hyperoxia and frequently experience arterial oxygen desaturations, we examined the combined effects of intermittent hypoxia with malnutrition on the developing lungs.

### Material and methods

This study was approved by the State University of New York, Downstate Medical Center Animal Care and Use Committee, Brooklyn, NY. Animals were cared for and handled according to the United States Department of Agriculture (USDA) guidelines. Euthanasia was carried out according to the American Veterinary Medical Association Panel for Euthanasia guidelines.

#### *Experimental design*

Certified infection-free, timed-pregnant Sprague Dawley rats were purchased from Charles River Laboratories (Wilmington, MA) at 18 days gestation. The animals were housed in an animal facility with a 12-hour-day/12-hour-night cycle and provided standard laboratory diet and water ad libitum. Within approximately 5 hours of birth, newborn rat pups delivering on the same day were pooled and randomly assigned to expanded litters of 18 pups/litter. The expanded litter size was used to simulate relative postnatal malnutrition in ill ELBW infants. Each pup was weighed and measured for linear growth (crown to rump length, cm) prior to experimentation. Ten groups were studied, 5 in normoxia (room air, RA), and 5 in intermittent hypoxia (IH) cycling. Newborn rat pups were separated from their mothers from the first day of life (P0) and kept at 37°C using a water-jacketed heating pad attached to a pump (Gaymar Industries, Orchard Park, NY). Group 1 received Similac, Advance infant formula with iron, ready to feed, 20 cal/fl oz with special nutrients found in breast milk and used as an alternative to breast milk from Ross/Abbot Labs (Form-Fed). Group 2 received Similac, Advance infant formula with iron supplemented with “Florastor Kids”, the # 1 Probiotic Worldwide made of live, freeze-dried yeast cells of the species *Saccharomyces boulardii* Iyo (5 mg/mL) from Biocodex, Inc. San Bruno, CA (Pro-Fed). Group 3 received Similac Advance Early Shield with iron, ready to feed, 20 cal/fl oz with prebiotics (galactocoligosaccharides, GOS & fructo-oligosaccharides, FOS) from Ross/Abbott Labs (Pre-Fed). Group 4 received Similac Advance Early Shield supplemented with 5 mg/mL *Saccharomyces boulardii* Iyo (Syn-Fed). Group 5 (control) was maternally fed (Mat-Fed) using the same dam throughout the study. The formula fed groups (Groups 1-4) were adminis-

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tered 0.2 mL formula every 3 hours using a 1 cc syringe attached to a feeding needle from P0 to P2 prior to euthanasia on P3. The pups were weighed and measured daily for changes in weight.

### *Intermittent hypoxia*

On the day of birth (P0), all IH-exposed litters were placed with the dams into specialized O<sub>2</sub> chambers (BioSpherix, New York) attached to an oxycycler. The oxycycler sensed O<sub>2</sub> inside the chamber and infused nitrogen to reduce O<sub>2</sub> and O<sub>2</sub> to raise it. Oxygen content inside the chamber was continuously monitored and recorded on a Dell Computer. The animals remained undisturbed except during the bedding change which occurred every 3 days, during a 50% O<sub>2</sub> cycle, and lasted no more than 3 minutes. The chamber was optimized for gas efficiency and provided adequate ventilation for the animals in a controlled atmosphere. Carbon dioxide in the chamber was continuously monitored and controlled with the use of a built-in fan and soda lime. Humidity in the chamber was also continuously monitored and controlled. At the start of the experiment, the IH profile consisted of 50% O<sub>2</sub> for 30 minutes followed by 3 clusters of 12% O<sub>2</sub> for 1 minute.

### *Sample collection*

On P3, animals were euthanized by decapitation and samples from the left lung were collected for histological (H&E stains), biochemical (VEGF, sVEGFR-1, dehydroepiandrosterone or DHEA, and corticosterone), and molecular (VEGF signaling) analyses. For histology and light microscopy, the lungs were inflated prior to fixation in 10% neutral-buffered formalin. The specimens were shipped to New York University Experimental Pathology Histology Core Laboratory, NY for tissue processing according to standard laboratory techniques. For biochemical assays, samples (approximately 200 mg) were rinsed in ice-cold phosphate buffered saline (PBS) and then placed in a clean polypropylene tube containing 2.0 mL sterile normal saline on ice. The tubes were then frozen at -20°C until assay. For real-time PCR arrays, 100 mg samples were biopsied from the left lung, rinsed in ice-cold sterile normal saline to remove blood elements, placed in RNase/DNase-free PCR tubes and frozen immediately at -80°C.

### *Assay of VEGF and sVEGFR-1*

On the day of the assay, lung samples were placed in individual tubes containing ceramic beads and homogenized using a Fast-Prep instrument (MP Biomedicals, Solon, OH) in 1.0 mL ice-cold sterile normal saline. The homogenates were centrifuged at 8,000 rpm for 20 minutes at 4°C and filtered prior to assay. VEGF and sVEGFR-1 levels in the homogenates were assayed using commercially-available sandwich immunoassay kits for rat/mouse from R & D Systems, Minneapolis, MN, USA and Diagnostic Systems Laboratories, Webster, TX, respectively, according to the manufacturer's protocol and standardized using total cellular protein levels.

### *Assay of DHEA and corticosterone*

DHEA and corticosterone levels were assayed in the lung homogenates using enzyme immunoassay kits purchased from Enzo Life Sciences, Plymouth Meeting, PA, according to the manufacturer's protocol. Tissue levels were standardized using total cellular protein levels in the samples.

### *Total cellular protein levels*

A 10 µL portion of the lung homogenates was utilized for total cellular protein levels using the Bradford method (Bio-Rad, Hercules, CA) with bovine serum albumin as a standard. The protein assay was carried out on the same day as the assays.

### *Histology of the lungs*

Samples were taken from randomly chosen rats from each group and fixed in 10% neutral buffered formalin and processed in paraffin. Five specimens per group were paraffin-embedded and blocks were cut in 5 µm sections and mounted on superfrost slides. There were 5 slides per block and each slide had 3 sections for a total of 75 sections per group. The slides were stained with Harris hematoxylin and phloxin B. Images were captured using an Olympus BX53 microscope, DP72 digital camera, and CellSens imaging software (Olympus, Center Valley, PA), attached to a Dell Precision T3500 computer (Dell, Round Rock, TX). Images of the slides were digitized at 640x480 pixels and 20x magnification.

### *Real-time polymerase chain reaction*

Total RNA was extracted using RNA Pro solution (MP Bio, Solon, Ohio) and allowed to digest for 5 minutes at room temperature. The samples were transferred to microtubes containing ceramic beads and placed in a FastPrep-24 instrument (MP Bio) for 40 seconds. The samples were centrifuged at 13,000 rpm at 4°C for 5 minutes, and the supernatant transferred to a clean Eppendorf tube. After addition of chloroform, the samples were vortexed for 30 seconds and centrifuged at 13,000 rpm at 4°C for 5 minutes. The upper aqueous phase was transferred to a clean Eppendorf tube containing 0.5 mL of ice-cold 100% ethanol. The samples were placed in a -20°C freezer for 60 minutes to precipitate the RNA. Following precipitation, the samples were centrifuged at 13,000 rpm at 4°C for 20 minutes and the resulting pellet was washed in 75% ethanol/water and redissolved in 100 µL nuclease-free water. The amount of RNA was quantified at 260 nm using a Beckman spectrophotometer and diluted to 1 µg/µL. Cleanup of the RNA was performed using RNEasy mini cleanup kits (Qiagen, Valencia, CA) followed by on-column treatment with DNase I (Qiagen). Reverse transcriptase was performed using a RT<sup>2</sup> First Strand kit purchased from SABiosciences, Frederick, MD. The real-time PCR arrays were carried out in duplicate with the resulting cDNA using the Rat Angiogenesis PCR Array System (a pathway focused gene expression profiler) on 96-well plates pre-coated with 84 genes regulating angiogenesis using a BioRad IQ5 real-time instrument (BioRad, Hercules, CA). The PCR cycle consisted of 1 cycle at 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. An aliquot of 25 µL sample in PCR master mix was added to each well. Each kit consisted of a panel of 5 housekeeping genes to normalize the PCR array data; replicate genomic DNA controls (GDC) to detect non-transcribed genomic DNA contamination with a high levels of sensitivity; replicate reverse transcription controls (RTC) to test the efficiency of the RT<sup>2</sup> first strand reaction; and replicate positive PCR controls (PPC) to test the efficiency of the PCR reaction itself using a pre-dispensed artificial DNA sequence and the primer set that detects it. The replicate controls also tests for inter-well and intra-plate consistency. Calculations were made by exporting the

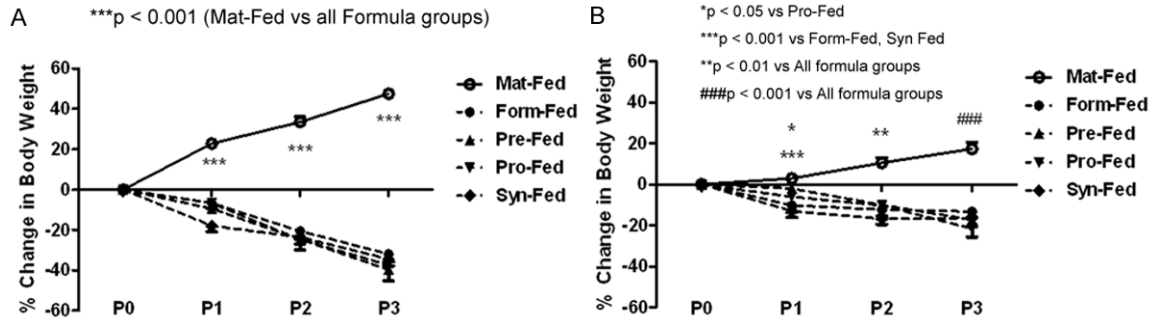
data into an Excel spreadsheet using the SABiosciences PCR Array Data Analysis Excel Template and uploading the real-time amplification data into the SABiosciences RT<sup>2</sup> Profiler PCR Array Data Analysis web portal. The PCR Array Data Analysis Web Portal automatically performs the calculations and interpretation of the control wells. Quantitative PCR was based on the cycle threshold ( $C_t$ ) value. The GDC in each PCR array specifically tests for genomic DNA contamination in each sample during each run. A GDC  $C_t$  value < 35 indicates the presence of a detectable amount of genomic DNA contamination. A value > 35 indicates that the level of genomic DNA contamination is too low to affect gene expression profiling results. None of the samples tested positive for contaminated genomic DNA. To determine if there were impurities in the sample that affected the reverse transcription phase, the following formula was used:  $\Delta C_t = \text{average } C_t^{\text{RTC}} - \text{average } C_t^{\text{PPC}}$  value. A value of > 5 was evidence of impurities in the sample that affected the reverse transcription phase. None of the samples tested positive for impurities. To determine if there were impurities in the sample that affected the PCR amplification phase, variations in the average  $C_t^{\text{PPC}}$  value were determined. A value of > 22 in the average  $C_t^{\text{PPC}}$  values between samples indicated the presence of different amounts of PCR amplification inhibitors. The average  $C_t^{\text{PPC}}$  value was less than 20 for each sample. A gene was considered not detectable if the  $C_t$  value was  $\geq 35$ . The  $\Delta C_t$  for each gene was calculated as  $C_t$  (gene of interest) -  $C_t$  (housekeeping gene).

### *Statistical analysis*

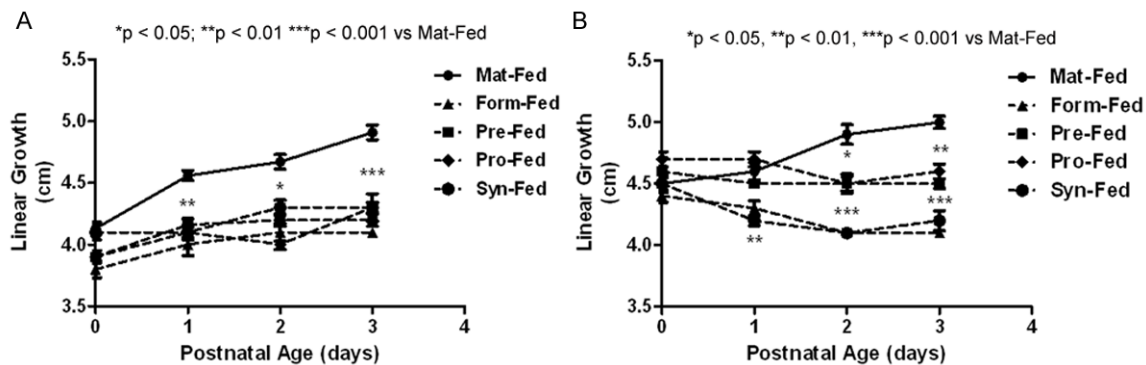
Unpaired t-tests or Mann Whitney U tests were used to determine differences between RA and hyperoxia groups following Levene's test for equality of variances. One-way analysis of variance was used to determine differences among the treatment groups for normally-distributed data, and Kruskal-Wallis test was used for non-normally-distributed data following Bartlett's test for equality of variances. Post hoc analysis was performed using the Tukey and Student-Newman-Keuls test for significance, as appropriate. Significance was set at  $p < 0.05$  and data are reported as mean  $\pm$  SEM. All analyses were two-tailed and performed using SPSS software version 16.0 (SPSS Inc., Chicago, IL) and GraphPad Prism software version 5.02 (GraphPad Inc., San Diego, CA).



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**Figure 1.** Percentage change in body weight in formula fed and maternally fed (Mat-Fed) neonatal rats raised in RA (A) or 50%/10% O<sub>2</sub> (B). Formula fed rats were non-supplemented (Form-Fed) or supplemented with prebiotics (Pre-Fed), probiotics (Pro-Fed), or synbiotics (Syn-Fed) (n=18/group on P0).



**Figure 2.** Changes in linear growth. Groups are as described in Figure 1.

## Results

### Effect on growth

Randomization at birth resulted in comparable mean total body weight (grams) and linear growth (cm) in all groups. All animals survived at P1. Mean body weight in RA was significantly lower in the Form-Fed ( $5.9 \pm 0.1$ ,  $p < 0.01$ ), Pro-Fed ( $6.0 \pm 0.08$ ,  $p < 0.01$ ), Pre-Fed ( $5.9 \pm 0.1$ ) and Syn-Fed ( $6.0 \pm 0.15$ ,  $p < 0.01$ ) groups compared to Mat-Fed ( $7.74 \pm 0.1$ ). Mean linear growth was similarly suppressed in the Form-Fed, Pro-Fed, Pre-Fed and Syn-Fed groups ( $4.0 \pm 0.09$ ;  $4.1 \pm 0.04$ ;  $4.2 \pm 0.04$ ; and  $4.1 \pm 0.06$ ,  $p < 0.01$ , respectively) compared to Mat-Fed ( $4.6 \pm 0.04$ ). By postnatal day 2 and 3, the mortality rate was higher in the Form-Fed group compared to the supplemented formula groups but the differences were not statistically significant. Weight loss continued to be significantly higher in all formula fed groups on P2 and P3 compared to Mat-Fed (Figure 1A). In hyperoxia, percentage change in weight loss was less dra-

matic in the formula-fed groups and a significantly greater number of animals survived on the day of euthanasia. We also noted higher weight loss in the maternally-fed group (Figure 1B). Mean linear growth remained lower in all formula groups at P2, and P3 compared to Mat-Fed (Figure 2A). In hyperoxia, differences among the groups were less significant and occurred only in the unsupplemented formula and Syn-Fed groups on P1, P2 and P3 compared to Mat-Fed. Pre-Fed and Pro-Fed were lower only on P2 and P3 (Figure 2B). In RA, lung weights were decreased in all formula groups compared to the Mat-Fed group (Figure 3A), while in hyperoxia, lung weights were preserved in the formula-fed supplemented groups (Figure 3B).

### Effects on VEGF and sVEGFR-1 levels

In RA, lung VEGF levels (pg/mg protein) were suppressed in the Form-Fed ( $6.7 \pm 1.3$ ,  $p < 0.001$ ), Pre-Fed ( $1.8 \pm 1.7$ ,  $p < 0.001$ ), and Syn-

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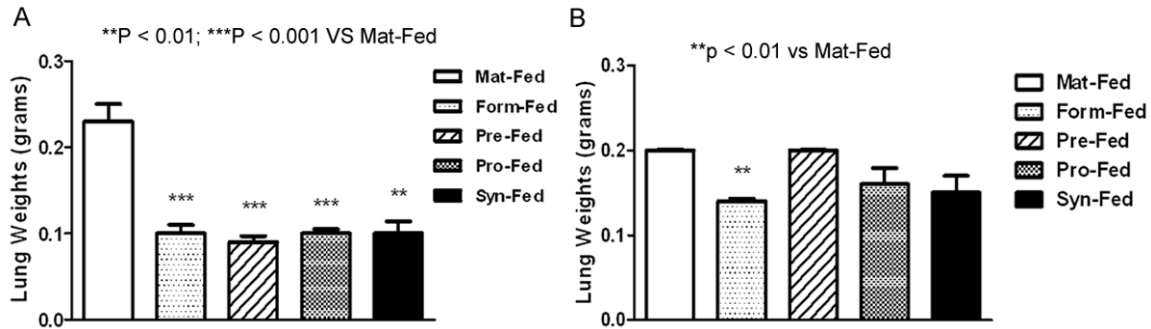


Figure 3. Mean lung weights. Groups are as described in Figure 1.

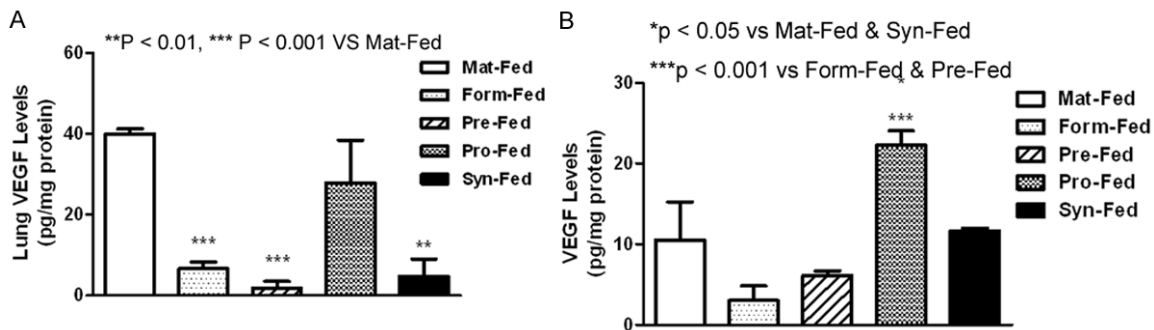


Figure 4. Mean lung VEGF levels. Groups are as described in Figure 1.

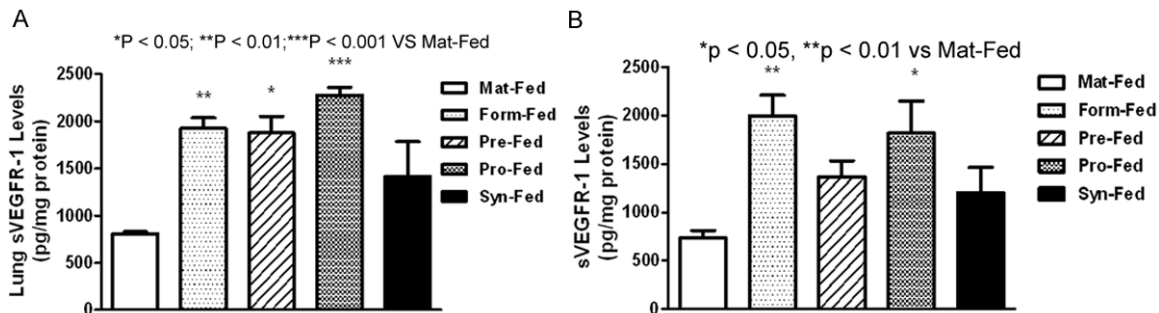


Figure 5. Mean lung sVEGFR-1 levels. Groups are as described in Figure 1.

Fed ( $4.8 \pm 4.2$ ), but not Pro-Fed ( $27.9 \pm 12.5$ ) groups compared to Mat-Fed ( $40.0 \pm 1.3$ ) (Figure 4A). In 50%  $O_2$ , lung VEGF was suppressed in all groups except Pro-Fed ( $22.3 \pm 1.8$ ,  $p < 0.05$ ) compared to Mat-Fed ( $10.5 \pm 4.7$ ) (Figure 4A). Figure 5A shows that lung sVEGFR-1 levels in RA were increased in all formula-fed groups, but significance was achieved in the Form-Fed ( $1921.9 \pm 109.4$ ,  $p < 0.01$ ), Pre-Fed ( $1875.0 \pm 173.0$ ,  $p < 0.05$ ), and Pro-Fed ( $2274.2 \pm 81.7$ ,  $p < 0.001$ ), but not Syn-Fed ( $1414.5 \pm 367.1$ ) compared to Mat-Fed ( $807.8 \pm 20.8$ ) groups. Similar elevations in lung sVEGFR-1 levels (pg/mg

protein) were noted in hyperoxia with significance in the Form-Fed ( $1995.3 \pm 213.0$ ,  $p < 0.01$ ) and Pro-Fed ( $1816.3 \pm 331.1$ ,  $p < 0.05$ ), but not Pre-Fed ( $1366.7 \pm 163.9$ ) and Syn-Fed ( $1200.7 \pm 262.8$ ), compared to Mat-Fed ( $734.2 \pm 78.2$ ) (Figure 5B).

### Effect on lung DHEA and corticosterone levels

Premature infants who develop CLD have elevated levels of cortisol precursors and lower cortisol values compared to infants who recovered [25]. We therefore measured the levels of

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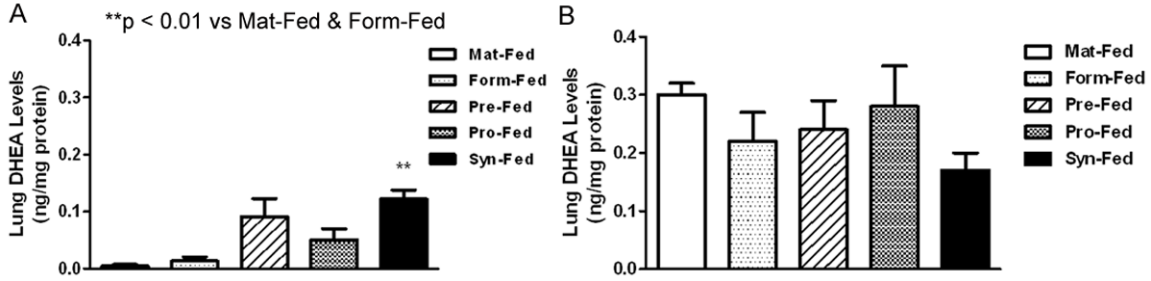


Figure 6. Mean lung DHEA levels. Groups are as described in Figure 1.

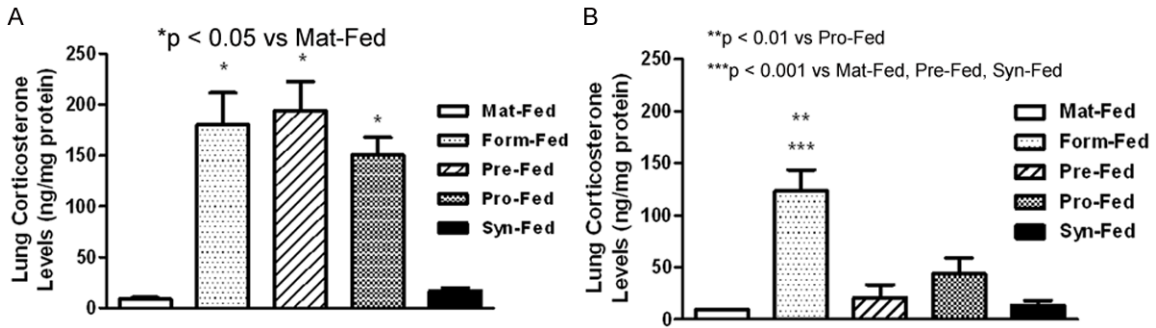


Figure 7. Mean lung corticosterone levels. Groups are as described in Figure 1.

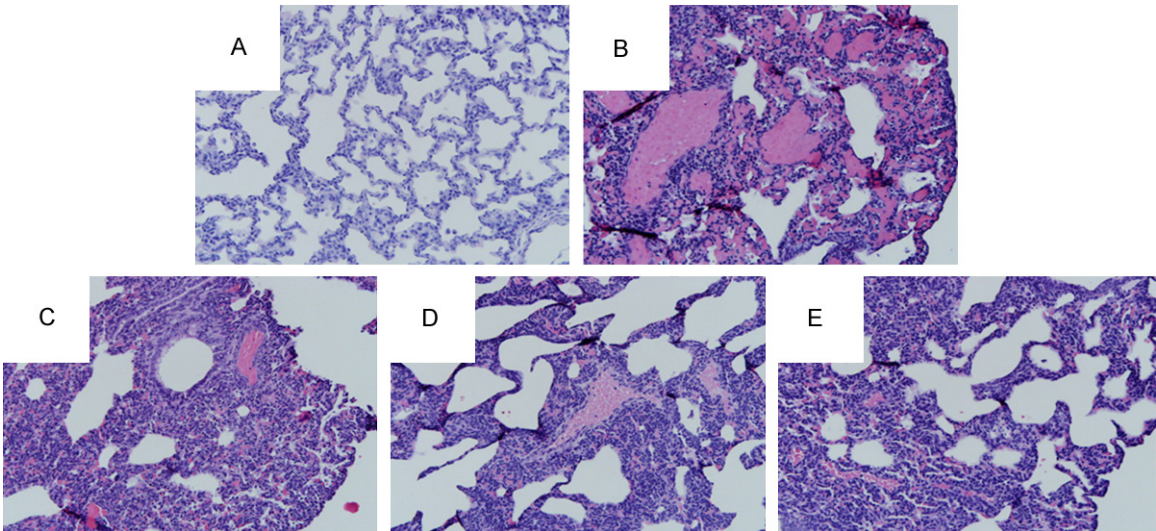


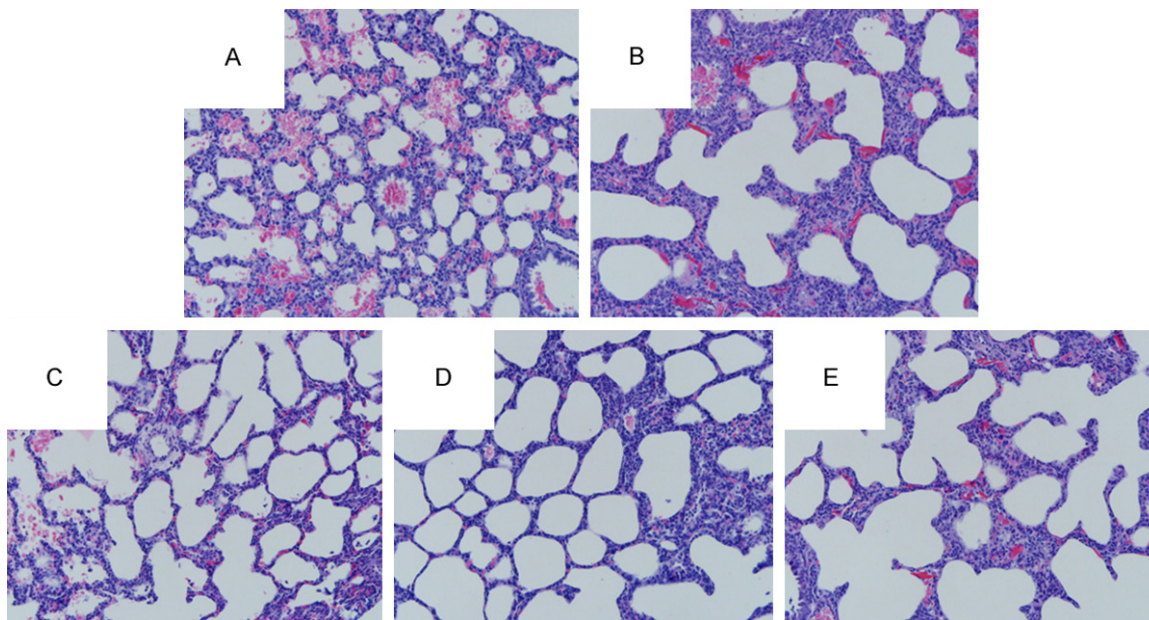
Figure 8. Representative histopathology of the lungs from maternally fed and formula fed pups raised in room air (H & E stain, 20X magnification). Lung architecture of a rat pup from the maternally-fed (A) shows normal alveoli. Unsupplemented formula fed rats (B) and rats supplemented with prebiotics (C), probiotics (D) and synbiotics (E) showed evidence of interstitial edema, atelectasis, decreased alveolarization, and disorganization of lung architecture.

DHEA and corticosterone in the rat lungs. In RA, DHEA levels (ng/mg protein) was high only in the Syn-Fed group ( $0.11 \pm 0.01$ ,  $p < 0.01$ ) compared to Mat-Fed ( $0.004 \pm 0.003$ ) (Figure

6A). In hyperoxia, DHEA values increased and were comparable among the groups (Figure 6B). Lung corticosterone levels (ng/mg protein) increased in the Form-Fed ( $180.0 \pm 31.5$ ,  $p <$



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**Figure 9.** Representative histopathology of the lungs from maternally fed and formula fed pups raised in intermittent hypoxia (H & E stain, 20X magnification). Lung architecture of a rat pup from the maternally-fed (A) shows normal alveoli with intraalveolar hemorrhage. Unsupplemented formula fed rats (B) shows evidence of interstitial edema, hemorrhage, decreased alveolarization, and disorganization of lung architecture. Treatment with prebiotics (C), probiotics (D) and synbiotics (E) showed less hemorrhage and more alveolarization, but the alveoli appear distended and oversimplified with less secondary crest formation.

0.05), Pre-Fed ( $193.8 \pm 28.8$ ,  $p < 0.05$ ), and Pro-Fed ( $150.5 \pm 17.3$ ,  $p < 0.05$ ) compared to Mat-Fed ( $8.9 \pm 1.5$ ) and Syn-Fed ( $16.8 \pm 3.0$ ) groups (**Figure 7A**). In hyperoxia, lung corticosterone was elevated only the Form-Fed ( $125.3 \pm 20.2$ ,  $p < 0.01$ ) compared to all other groups (**Figure 7B**).

### Histology

Histological analysis of the lungs revealed that compared to 3-day old Mat-Fed RA controls, all formula-fed pups had evidence of interstitial edema, intra-alveolar hemorrhage, atelectasis and disorganization of lung architecture (**Figure 8B-E**). In contrast, hyperoxia resulted in the appearance of more mature lungs however there was significant hemorrhage in the Mat-Fed, Form-Fed and Pre-Fed groups (**Figure 9A-C**, respectively). Although no hemorrhage was noted in the Pro- and Syn-Fed groups, the alveoli appeared uniformly distended and oversimplified with less evidence of secondary crest formation (**Figure 9D and 9E**).

### Effect of genes regulating angiogenesis

**Table 1** shows the fold regulation of several genes involved in lung VEGF signaling and

angiogenesis in RA formula-fed pups compared to maternally-fed pups. In the Form-Fed group there was a 7-fold downregulation of collagen type IV accompanied by upregulation of MMP-3 (4-fold), MMP-9 (7-fold), and TIMP-3 (3-fold). VEGF and VEGFR-2 were downregulated 2-fold, while VEGFR-1 was upregulated 3-fold. In the Pre-Fed group we noted 149-fold, 209-fold, and 17-fold increases in leptin, MMP-3, and MMP-9, respectively, while MMP-2 (8-fold), NP-2 (13-fold), and VEGFR-2 (26-fold) genes were upregulated. Supplementation with probiotics and synbiotics had more moderate effects. In hyperoxia (**Table 2**), the major findings were 44-fold upregulation in MMP-9 in the Form-Fed group concurrent with 12-fold and 4-fold downregulation in VEGF and VEGFR-2 genes. Probiotics appeared to be more beneficial for lowering MMP-9 upregulation (2-fold) compared to prebiotics (17-fold) and synbiotics (12-fold). In addition, HIF<sub>1 $\alpha$</sub>  was upregulated 12-fold in the Form-Fed group compared to the supplemented groups (1-fold upregulation).

### Discussion

The neonatal rat model has been validated as a good model for the study of NEC with similar



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**Table 1.** Real-time PCR profile of genes involved in angiogenesis in normoxic, formula-fed neonatal rat lungs. Data are fold change from maternally-fed rat lungs

Genes of Interest	Form-Fed	Pre-Fed	Pro-Fed	Syn-Fed
ColIV	-6.6	-3.1	-1.2	-4.3
HIF <sub>1α</sub>	-1.4	-1.4	1.9	-1.4
IGF-1	-2.3	-1.6	-2.9	-3.4
Lep	3.1	149.0	2.9	3.2
MMP-2	-1.1	-8.0	1.5	-3.0
MMP-3	3.6	209.2	24.1	6.9
MMP-9	6.8	16.6	7.8	8.4
NP-1	-4.0	1.3	1.0	-1.0
NP-2	-4.0	-13.1	-3.0	-5.2
PECAM-1	-1.4	-6.7	1.2	-1.5
TIMP-1	-1.3	-2.1	1.2	-2.3
TIMP-2	-1.2	-7.0	1.6	1.5
TIMP-3	3.3	-3.4	5.0	2.0
VEGF-A	-1.6	3.0	1.2	1.1
VEGFR-1	2.8	2.4	3.9	2.2
VEGFR-2	-2.4	-25.8	-4.2	-1.8

All data were corrected using 5 different housekeeping genes. Genes are selected from a profile of 84 genes. In alphabetical order the genes of interest are: ColIV (collagen type IV); HIF<sub>1α</sub> (hypoxia-inducible factor-α); IGF-1 (insulin-like growth factor-1); Lep (leptin); MMP (matrix metalloproteinase); NP (neuropilin); PECAM-1 (platelet/endothelial cell adhesion molecule-1); TIMP (tissue inhibitor of metalloproteinase); VEGF (vascular endothelial growth factor); VEGFR (VEGF receptor).

characteristics as human NEC [26-28]. In addition to NEC from enteral feeding, our model examined the effects of two stressors: 1) rat pups were separated from their mothers on the first day of life and administered a measured amount of formula on a fixed schedule, compared to controls which remained with their mothers and fed ad libitum; and 2) IH. Formula-fed rat pups did not adapt well to the scheduled feeding regiment evidenced by the poor weight accretion, abdominal distension and delayed gastric emptying. The results showed a significant difference in weight gain between the maternally-fed groups in RA and IH, compared to the other groups despite supplementation with prebiotics, probiotics or synbiotics, suggesting gross malnutrition.

Nutrition is important for normal lung development [29]. Animal studies have shown that early malnutrition alters surfactant and delays lung repair [30]. Growth failure precedes and follows CLD in preterm infants, persists throughout hospitalization, and is associated

with the development of BPD [31]. In our study, all RA pups had significant lung growth. The changes in lung parenchyma and general architecture in response to malnutrition seen in the present study was consistent with previous studies in rats [32] and lungs of severely undernourished patients [33]. We also noted that lung size was markedly lower in the formula-fed groups suggesting reduced alveolar number and surface area and confirmed by histology. Our findings are in support of providing adequate nutrition to premature infants in order to improve lung architecture and function [1, 27, 34]. Furthermore, our findings suggest that in the setting of NEC, supplementation may improve pulmonary status.

VEGF is a potent endothelial cell mitogen that plays a fundamental role in normal and pathologic angiogenesis. In the lungs VEGF is synthesized by alveolar epithelial cells, epithelial bronchial cells, smooth muscle cells and alveolar macrophages [35] and is necessary for microvascular maturation and efficient blood-air interface. Decreased VEGF levels in the lungs contribute to the development of BPD [36, 37]. VEGF binds and mediates its actions via receptors present on endothelial cells. Knockdown of VEGFR-1 and VEGFR-2 arrests lung growth and leads to emphysema [38]. In the present study, VEGF protein was preserved with probiotics, and VEGF mRNA was upregulated in all supplemented formula groups. Despite this, there was histological evidence of lung damage in all formula-fed RA groups. In contrast, sVEGFR-1 protein was high in all formula-fed groups as was VEGFR-1 mRNA. This may suggest that in this model, sVEGFR-1 is a more important marker for lung damage associated with all the hallmarks of BPD (i.e. emphysema, inflammation, fibrosis and arrested lung development) as previously demonstrated [24]. VEGFR-2 was downregulated in all formula-fed groups particularly in the Pre-Fed group (26-fold). Since VEGFR-2 binding to VEGF and NP-1 mediates potent angiogenesis, this finding suggests arrested lung VEGF signaling with prebiotics in the setting of NEC, malnutrition and/or oxidative stress.

Matrix metalloproteinases (MMPs), are a family of zinc-dependent endopeptidases that breakdown extracellular matrix (ECM) to enhance angiogenesis. MMPs are regulated by tissue inhibitor of metalloproteinases (TIMPs) and a balance between MMPs and TIMPs is neces-

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**Table 2.** Real-time PCR profile of genes involved in angiogenesis in hyperoxic, formula-fed neonatal rat lungs. Data are fold change from maternally-fed rat lungs

Genes of Interest	Form-Fed	Pre-Fed	Pro-Fed	Syn-Fed
ColIV	-1.8	-3.5	-1.1	-1.9
HIF <sub>1α</sub>	12.0	1.2	1.4	1.2
IGF-I	1.3	-1.6	-1.5	1.7
Lep	-2.5	-3.5	2.5	-1.2
MMP-2	-2.0	-3.2	-1.7	-1.5
MMP-3	1.4	-3.2	8.1	2.6
MMP-9	44.0	17.0	2.0	12.0
NP-1	-4.6	-2.3	-1.3	-1.8
NP-2	-4.8	-3.6	-1.0	-1.0
PECAM-1	-1.3	-1.8	-1.1	1.3
TIMP-1	2.1	-1.5	2.1	2.0
TIMP-2	-3.8	-1.3	1.2	1.5
TIMP-3	-3.6	-1.6	3.4	3.1
VEGF-A	-12.3	-1.6	-1.2	-1.5
VEGFR-1	-2.3	-1.3	1.6	-1.1
VEGFR-2	-4.2	-2.0	-1.3	-1.2

The genes of interest are as described in **Table 1**.

sary for harmonious ECM remodeling. MMP-3 activates proMMP-9 (precursor of MMP-9), a known mediator of CLD [39]. High MMP-3 and -9 and low MMP-2 levels are associated with lung inflammation and injury [23, 40-42]. MMP-2 expression was preserved only with probiotics. MMP-9 was upregulated in all formula-fed groups in RA and O<sub>2</sub> confirming lung inflammation. However, we were surprised to find robust upregulation of MMP-3 with prebiotics in RA suggesting increased activation of proMMP-9. Despite hyperoxia, the lung HIF<sub>1α</sub> was upregulated in the For-Fed group indicating hypoxia. This was paralleled by robust upregulation of MMP-9 and downregulation of VEGF and its two membrane receptors. Only probiotics ameliorated the MMP-9 response suggesting a beneficial effect on lung inflammation. Together, these findings support a role for probiotic supplementation to reduce lung injury secondary to NEC and malnutrition in the setting of oxidative stress.

Long term separation from the mother is a known risk factor for stress in rats [43]. Early life experiences, such as the maternal bonding, tactile stimulation, licking and grooming the pups are important to regulate their HPA axis and promote behavioral and physiological

responses to stress [44]. Cortisol helps facilitate the body's response to stress and regulates the immune system to minimize the effects of inflammation. Preterm infants who develop CLD have lower cortisol and higher cortisol precursors [25]. Cortisol insufficiency predisposes preterm infants to inflammation. In our experiment, maternal separation caused low DHEA and high corticosterone levels in all RA groups except the Syn-Fed group. This suggests that the use of synbiotics is associated with a decreased capacity to synthesize corticosterone. Hyperoxia increased DHEA in all groups with subsequent reductions in corticosterone except in the Form-Fed group. Higher secretion of corticosterone and its precursor may indicate increased anxiety and stress in that group.

Our findings support the provision of adequate nutrition to preterm infants who experience frequent IH in order to improve lung architecture and function as suggested by others [1, 27, 32]. Our findings further suggest that in the setting of IH, supplementation with probiotics may improve pulmonary status. Our model supports the finding that elevated sVEGFR-1 is an important marker for lung damage [24]. The use of prebiotics was associated with arrested lung VEGF signaling, while probiotics ameliorated the MMP-9 response suggesting a beneficial effect on lung inflammation. Synbiotics was associated with a decreased capacity to synthesize corticosterone in our NEC/malnutrition model and histological findings showed distended and oversimplified alveoli. Together, these findings support a role for supplementation and provide the basis for further studies of with the use of probiotics to reduce lung injury secondary to IH, NEC and/or malnutrition.

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### Disclosure of conflict of interest

None to declare.

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