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Dietary (1,3/1,6)-β-D-glucan minimally impacts development of lung immunity in the neonatal piglet

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ABSTRACT

Identification and characterization of compounds that enhance the growth, development and health of infants who are not breastfed continues to be a goal for nutritional science. This study explored the effects of one dietary component, (1,3/1,6)-β-D-glucan (Wellmune WGP®) on lung immune development in the neonatal piglet. Piglets were fed a control formula or formula plus WGP at 1.8, 18 or 90 mg/kg BW/day. Serum, thoracic lymph nodes (TLN), mediastinal lymph nodes (MSLN) and lung were collected at day 7 or day 21. Immune parameters including tissue mRNA expression and T cell phenotypes were analyzed. Normal developmental changes were observed, with a decrease in T-helper cells and an increase in cytotoxic T cells in both TLN and MSLN, but there was no effect of WGP. Dietary WGP reduced the mRNA expression of TGFβ-2 and tended to reduce the mRNA expression of TGFβ-1 in lung tissue. With the exception of reducing TGFβ mRNA in lung and tending to decrease the T helper to cytotoxic T cell ratio in the TLN, dietary WGP did not affect lung-associated adaptive immunity in piglets.

Keywords: Beta-glucan; Piglet; Lung; Immune; Vaccination

Abbreviations: βG(s), β-Glucan(s); *BW,* body weight; *BSA,* bovine serum albumin; *CMI,* cell-mediated immune; *d,* day; *h,* hour; *H&E,* hematoxylin and eosin,; *MSLN,* mediastinal lymph nodes; *PBS,* phosphate buffered saline; *PRR(s),* pattern recognition receptor(s); *RT,* room temperature; *TLN,* thoracic lymph nodes; *TLR,* toll like receptors; *WGP,* Wellmune (1,3/1,6)-β-D-glucan

1. INTRODUCTION

Upon taking their first breath, the newborn is exposed to airborne pathogens. The first point of contact for inhaled substances is the epithelial lining of the upper airways and lungs [1]. Therefore, mucosal defense mechanisms are critical in preventing colonization of the respiratory tract by pathogens and penetration of antigens through the epithelial barrier. In recent years, it has become clear that airway epithelial cells not only function as a passive barrier, but also actively participate in innate immune responses [1,2]. Respiratory infections are a major cause death in infants and children worldwide [3]. Infants must rapidly develop immune protection to defend against infection, while avoiding harmful inflammatory responses and optimal nutrition is key to the development of the innate and adaptive immunity in the neonate [4]. Breastfeeding confers protection against respiratory infections in both developed [5,6] and developing countries [7], with infants who were exclusively breastfed for 4 to <6 months being 4.27 (95% confidence interval: 1.27-14.35) more likely to develop pneumonia than infants exclusively breastfed for ≥6 months [5]. However, recent statistics show that the current rates of exclusive or any breastfeeding fall below recommendations [8]. Therefore there remains a need to identify potential ingredients that will improve immune function of formula-fed infants.

βGs are polysaccharides that occur as a principal

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component of cellular walls in yeast, fungi, seaweeds, mushrooms, and some cereals such as oats and barley. They do not have direct cytotoxic activities, but are able to boost the natural defense mechanisms of the host. βG signals the immune system through pattern-recognition receptors (PRRs) present on immune cells. These PRRs include at least four receptors including dectin-1, complement receptor 3 (CR3; CD11b/CD18), lactosylceramide receptors, and scavenger receptors [9]. Through these receptors βGs stimulate the innate immune system, modulate humoral and cellular immunity, and may improve an individual's ability to fight infections [10-13].

Airway epithelial cells participate in immune responses through PRR signaling, which results in the expression of a variety of immune-related genes. In fact, airway epithelial cells have been shown to secrete a large variety of molecules that are involved in inflammatory and immune processes including cytokines, chemokines, leukotrienes, calprotectin, β-defensins and other factors [1,2]. Through the secretion of these substances, the airway epithelium attracts and activates cells of the innate immune system. These cells subsequently immobilize and kill microorganisms, induce wound healing and angiogenesis in response to injury and orchestrate the initiation of adaptive immune responses [1].

A previous study demonstrated a reduction in pulmonary lesion score and viral replication rate of pneumonia induced by swine influenza virus in young piglets fed *Saccharomyces cerevisiae (S. cerevisiae)* βG (50 mg/day) [14], suggesting the potential for dietary yeast βG to exert activity in the lung. However, minimal research has been done to explore the effects of βG on immune development of the lungs in very young animals or humans. Herein we tested a clinically relevant βG, (1,3/1,6)-β-D-glucan (Wellmune WGP®, WGP), which is generally recognized as safe by the United States FDA, on the development of lung-associated adaptive immunity in piglets less than three weeks of age. We hypothesized that the addition of dietary WGP would alter the development of lung immunity in the neonatal piglet. Specifically, WGP supplementation would increase inflammatory cytokine production.

2. MATERIALS AND METHODS

2.1 Chemicals

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise indicated.

2.2 Dietary Yeast β-Glucan

(1,3/1,6)-β-D-glucan (Wellmune WGP®, WGP) was obtained from Biothera, Inc. (Eagan, MN). This compound was extracted from *S. cerevisiae* using a process that produces a whole glucan particle in which the outer surface of mannoprotein and inner cellular contents are removed [15]. WGP existed as a particulate suspension in the bovine milk-based formulas used herein. WGP was supplemented to formula to provide doses at 1.8mg/kg BW/day, 18mg/kg BW/day or 90mg/kg BW/day. The lowest dose provided an average WGP intake of 5 mg/day, slightly exceeding Biothera's recommendation of 2 mg/kg BW/day. The middle dose provided an average WGP intake of 50 mg/day. The highest dose provided an average WGP intake of 250 mg/d. The two lowest doses do not surpass the level generally recognized as safe by the United States FDA (200mg/serving, GRN No. 239, www.FDA.gov accessed July 16, 2012). Furthermore, these levels are within the range that has been shown to result in no observed adverse effects in toxicological testing (2-100 mg/kg BW/day) [15].

2.3 Dietary Treatment and Animal Protocol

Piglets (n=68) were obtained at 48 h postpartum to allow for consumption of colostrum. The piglets were randomized to one of four dietary treatment groups: 1) a medicated sow milk replacer formula (WGP0) (Formula; Milk Specialties Global Animal Nutrition, Carpentersville, IL); 2) Formula + 5 WGP mg/L (WGP1.8); 3) Formula + 50 WGP mg /L (WGP18); or 4) Formula + 250 WGP mg/L (WGP90). Piglets were individually housed in environmentally controlled rooms (25°C) in cages capable of maintaining six piglets separated by Plexiglas partitions. Radiant heaters were attached to the tops of the cages to maintain an ambient temperature of 30°C. Formula was offered 14 times daily at a rate of 360 ml/kg BW/day. The piglets were monitored daily for normal growth and food intake, as well as the presence of fever, diarrhea or lethargy. All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Illinois.

2.4 Sample Collection

On day 7 (WGP0 n=5, WGP1.8 n=5, WGP18 n=5, WGP90 n=5) or day 21 (WGP0 n=12, WGP1.8 n=13, WGP18 n=11, WGP90 n=12) postpartum, piglets were sedated with an intramuscular injection of Telazol® (tiletamine HCl and zolazepam HCl, 3.5 mg/kg BW each,

Pfizer Animal Health, Fort Dodge, IA). After sedation, blood was collected by cardiac puncture into non-coated vacuum vials for serum isolation. Piglets were then euthanized by an intravenous injection of sodium pentobarbital (Fatal Plus, Vortech Pharmaceuticals, Dearborn, Michigan; 72mg/kg BW). After death, a thoracotomy was performed and lungs, mediastinal lymph nodes (MSLN) and thoracic lymph nodes (TLN) were quickly excised. Sections (3-4 cm) of the lung were snap frozen in liquid nitrogen or fixed in Bouin's solution. MSLN and TLN were snap frozen in liquid nitrogen. The remaining MSLN and TLN were collected for isolation of cells. Additionally, intestine, spleen, mesenteric lymph nodes and peripheral blood mononuclear cells were collected, and data obtained from those tissues are presented in another manuscript, where intestinal and systemic immune development was unaffected by dietary WGP [16].

2.5. Lung Histomorphology and T cell Immunohistochemistry

Bouin's-fixed lung samples were embedded in paraffin, sliced to approximately 5µm with a microtome, and mounted on glass microscope slides. Slides were then stained with hematoxylin and eosin (H&E) or anti-human CD3 polyclonal antibody at the University of Illinois Veterinary Diagnostic Laboratory. Because the tissues were paraffin-embedded and Bouin's-fixed, antigen retrieval was performed. A citrate buffer (pH 6.0) was used to break protein crosslinks formed by fixation allowing the antibody to recognize the CD3 protein. After antigen retrieval, slides were incubated with a rabbit anti-human CD3 polyclonal antibody (Biocare Medical, Concord, CA). This antibody cross-reacts with pig CD3 and is extensively used by the Veterinary Diagnostic Histology Laboratory at the University of Illinois College of Veterinary Medicine (whose staff has verified this cross-reactivity by achieving the expected staining of pig lymph node sections). Staining was visualized using a HRP/DAB (horse-radish peroxidase/ diaminobenzidine) system (Super Sensitive Polymer-HRP Detection System, Biogenex, San Ramon, CA) following the manufacturer's instructions. Briefly, slides were blocked (casein/PBS) for 20min, secondary anti-rabbit-polymer-HRP antibodies were incubated with slides for 30min. HRP was visualized by DAB exposure for 5min. Slides were counter-stained with hematoxylin for 1min. The NanoZoomer Digital Pathology System was used to image slides (University of Illinois, Institute for Genomic Biology, Core Facilities). Images were then analyzed by a board certified veterinary pathologist at the University of Illinois College of Veterinary Medicine.

2.6 Isolation of Total Cells from Lymph Nodes

Mononuclear cells from TLN and MSLN were obtained by mechanical dissociation using a Gentle Macs Dissociator (Miltenyi Biotec, Auburn, CA). Cells were then sequentially passed through 100um and 40um cell strainers (BD Biosciences, Bedford, MA) to form single cell suspensions. Cells were counted using a Countess automated cell counter (Life Technologies, Invitrogen, Carlsbad, CA). The number of viable cells was assessed by trypan blue (Life Technologies, Invitrogen Gibco) exclusion. Isolated cells were kept in complete medium (RPMI 1620 (Invitrogen Gibco), 20% fetal calf serum (Invitrogen Gibco), 2 mM L-glutamine (Invitrogen Gibco), 50 µg/ml gentamicin (Invitrogen Gibco), 100 µg/ml penicillin, and 100 µg/ml streptomycin) until use.

Table 1. Primers Used for Quantitative Real Time-PCR

Gene	Forward Primer (5' to 3')	Reverse Primer (5' to 3')	Gene Bank Accession ID
β-actin	CACGCCATCCTGCGTCTGGA	AGCACCGTGTTGGCGTAGAG	DQ845171.1
Dectin	CTCTCACAACCTCACCAGGAGAT	CAGTAATGGGTCGCCAATAAGG	FJ386384.1
IL-2	TCAACTCCTGCCACAATGT	CTTGAAGTAGGTGCACCGT	EU139160.1
IL-12	CGTGCCTCGGGCAATTATAA	CAGGTGAGGTCGCTAGTTTGG	NM_213993.1
IL-4	CACAGCGAGAAAGAACTCGT	GTCCGCTCAGGAGGCTCTTC	NM214123.1
IL-6	CTGGCAGAAAACAACCTGAACC	TGATTCTCATCAAGCAGGTCTCC	AB194100.1
IL-1α	GTGCTCAAAACGAAGACGAACC	CATATTGCCATGCTTTTCCCAGAA	X52731.1
TNF-α	AACCTCAGATAAGCCCGTCG	ACCACCAGCTGGTTGTCTTT	EU682384.1
TGF-β1	CCTGCAAGACCATCGACATG	GCCGAAGCTTGGACAGAATC	AF461808.1
TGF-β2	TGTGTGCTGAGCGCTTTTCT	GAGCGTGCTGCAGGTAGACA	L08375.1

Table 2. T-lymphocyte Populations in Thoracic Lymph Nodes of 7- and 21-day old Piglets consuming 0 to 90 mg/kg/d of WGP

	WGP0[1]	**WGP1.8**	**WGP18**	**WGP90**
Day 7				
T-helper[2]	73.4±6.0	68.6±10.2	72.1±4.4	72.1±3.0[a]
Cytotoxic [3]	7.3±1.6	13.0±3.0	14.1±5.6	9.9±2.0[a]
Double Positive [4]	7.4±0.7	9.0±1.4	6.2±1.9	6.6±2.9
Day 21				
T-helper	66.4±9.4	68.8±6.6	65.7±5.6	68.7±4.0[b]
Cytotoxic	12.0±3.8	12.0±3.2	14.8±3.0	12.6±2.8[b]
Double Positive	9.9±5.1	10.4±5.3	8.4±1.1	8.2±1.4

Data are expressed as mean ± SD. Percentages within a T-lymphocyte subpopulation with different superscript letters are significantly different by day (general linear model, Fisher least-significant-difference post hoc testing [SAS 9.2]; $p<0.05$). There were no differences by diet.

[1] Numbers indicate dietary dose in milligrams per kilogram of BW per day.

[2] Values are $CD45^+CD3^+CD4^+CD8^-$ events as a percentage of $CD45^+CD3^+$ events

[3] Values are $CD45^+CD3^+CD8^+CD4^-$ events as a percentage of $CD45^+CD3^+$ events

[4] Values are $CD45^+CD3^+CD8^+CD4^+$ events as a percentage of $CD45^+CD3^+$ events

2.7 Phenotypic Identification of Cells

The phenotypes of T lymphocyte subpopulations from MSLN and TLN were monitored using fluorescently labeled mAbs. Lymphocytes were identified by anti-swine CD45 (Clone K252-1E4, AbD Serotec, Raleigh, NC). Anti-CD45 was conjugated to Alexa 647 with a Zenon Mouse Antibody Labeling Kit (Invitrogen Molecular Probes, Eugene, OR). T cells were identified by mouse anti-pig CD3:biotin (Clone BB23-8E6, Southern Biotech, Birmingham, AL) which was visualized with streptavidin:PE-Cy7 (Southern Biotech). To further differentiate T cell populations, cells were stained with mouse anti-pig CD4:FITC (Clone 74-12-4, Southern Biotech) and mouse anti-pig CD8:PE (Clone 76-2-11, Southern Biotech). All staining procedures took place on ice and care was taken to prevent unnecessary exposure to light. Briefly, one million cells per well were blocked with a mixture of 5% mouse serum (Southern Biotech) and 200µg/ml purified mouse IgG (Invitrogen) for 5min. Next, cells were incubated for 20min in a total of 10µl CD3:biotin. Cells were then centrifuged at 2000rpm, 5min, 4°C and supernatants were removed. Cells were then incubated for 20 min in a total volume of 40µl (10µl each: CD45, Strep-PECy7, CD4 and CD8). Cells were washed twice with phosphate buffered saline (PBS)/1% bovine serum albumin (BSA)/0.1% sodium azide, and then fixed with 2% paraformaldehyde. Staining was assessed using an LSRII flow cytometer (BD™, Biosciences, San Jose, CA). The relative number of T cell subpopulations was determined using FlowJo 7.0 software (FlowJo, Ashland, OR). $CD45^+CD3^+$ events were considered T cells. $CD45^+CD3^+CD4^+CD8^-$ events were considered T helper cells. $CD45^+CD3^+CD8^+CD4^-$ event were considered cytotoxic T cells. $CD45^+CD3^+CD4^+CD8^+$ events were considered double positive T cells.

2.8 Mitogenic Cell Stimulation

TLN and MSLN cells were plated in 96-well plates, 2 x 10^5 cells per well, in a final volume of 200 µl complete medium at 37°C under 5% CO_2. 20 µl of LPS (20 µg/ml) or 20 µl ConA (25 µg/ml) was added on d0 (n=3 wells per sample per stimulant). Plates were incubated for 72h, then pulsed with ^3H-thymidine (1 µCi/per well; Perkin Elmer, Boston, MA) and incubated for an additional 24h. Plates were stored at -80°C until analyzed. Cells were harvested (TomTech, Harvester 96 Mach III M, Hamden, CT) onto 1.5µm glass fiber filter paper (Skatron Instruments, Sterling, VA) and transferred into scintillation vials with 7ml Ultima Gold™ F scintillation fluid (Perkin Elmer, Boston, MA). Samples were counted on a Beckman Coulter, LS 6500 Scintillation System (Brea, CA). Data are expressed as a change in counts per minute (Δcpm) which was obtained by subtracting counts from unstimulated control wells from counts for wells with mitogens. Samples were analyzed in triplicate. Data analysis was done on log transformed Δcpm.

2.9 Tissue Cytokine mRNA Expression

Total RNA was extracted from snap frozen lung, TLN, and MSLN samples with TRIzol reagent (Invitrogen). RNA was quantified by spectrophotometry using a Nanodrop 1000 (Thermo Scientific, Rockford, IL) at absorbancy 260nm. RNA concentration was adjusted to 0.25µg/ml using RNase

Table 3. T-lymphocyte Populations in MSLNs of 7- and 21-day old Piglets consuming 0 to 90 mg/kg/d of WGP[1]

	WGP0	WGP1.8	WGP18	WGP90
Day 7				
T-helper[2]	74.0±7.0	75.2±2.7	70.2±14.1	75.1±2.7[a]
Cytotoxic [3]	7.7±1.9	8.4±3.7	13.1±4.0	7.6±1.3[a]
Double Positive [4]	6.2±2.9	7.2±2.9	7.7±1.8	7.6±2.9
Day 21				
T-helper	62.6±8.7	61.3±3.4	67.0±7.6	66.6±3.0[b]
Cytotoxic	15.6±2.9	16.9±2.4	14.2±2.0	17.3±2.1[b]
Double Positive	9.3±0.3	8.2±2.0	7.2±4.4	8.4±4.0

Data are expressed as mean ± SD. Percentages within a T-lymphocyte subpopulation with different superscript letters are significantly different by day (general linear model, Fisher least-significant-difference post hoc testing [SAS 9.2]; $p<0.05$). There were no differences by diet.

[1] Numbers indicate dietary dose in milligrams per kilogram of BW per day.

[2] Values are $CD45^+CD3^+CD4^+CD8^-$ events as a percentage of $CD45^+CD3^+$ events

[3] Values are $CD45^+CD3^+CD8^+CD4^-$ events as a percentage of $CD45^+CD3^+$ events.

[4] Values are $CD45^+CD3^+CD8^+CD4^+$ events as a percentage of $CD45^+CD3^+$ events.

free water (Invitrogen). RNA quality was assessed by a 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA). All samples had an RNA integrity number (RIN) greater than 6. Reverse transcription was performed on 3μg total RNA in a reaction volume of 20μl (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems, Foster City, CA). Quantitative real-time PCR was conducted using SYBR-Green (Roche Diagnostics GmbH, Mannheim, Germany) and data was collected using the Taqman ABI 7900 machine (Applied Biosystems Inc., University of Illinois, Keck Center for Comparative and Functional Genomics). A total of 40 PCR cycles were run. Primers used are listed in **Table 1** and final primer concentrations were 300nM. The relative standard curve method was used for quantification. Beta-actin was used as endogenous control. Standard curves from a stock of pooled porcine spleen cDNA was made and run on each plate. Normalized values for each target were calculated by dividing the target quantity mean by the β-actin quantity mean. Fold change was calculated for each measurement by dividing the normalized target values by the normalized calibrator sample (in this case the d21, FF group average). All samples that were statistically compared to each other were run on the same plate.

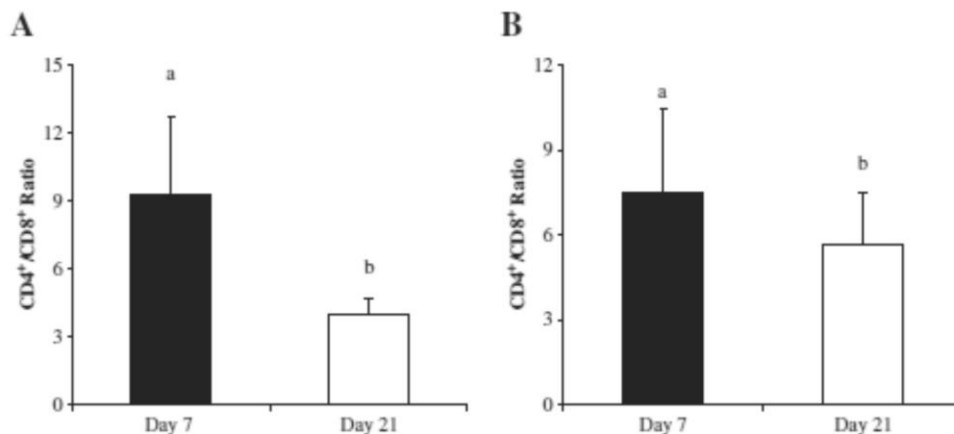

Fig. 1 – T-helper/cytotoxic T-cell ratio decreases in MSLNs and TLNs as piglets age. No diet (MSLN P = .30, TLN P = .07) differences were detected; therefore, data were pooled within day. MSLN (A; model P = .004, day P = .0001) and TLN (B; model P = .019, day P = .0140). For day 7, n = 17 samples per tissue. For day 21, n = 24 samples for TLN and 10 samples for MSLN. Data are expressed as the ratio of T helper to cytotoxic T cell (means ± SD) where T-helper-cell and cytotoxic T-cell events were originally expressed as a percentage of T (CD45+CD3+) lymphocytes. Statistical significance was determined using the general linear model, Fisher least-significant-difference post hoc testing (SAS 9.2; P < .05). Different letter superscripts indicate significant differences.

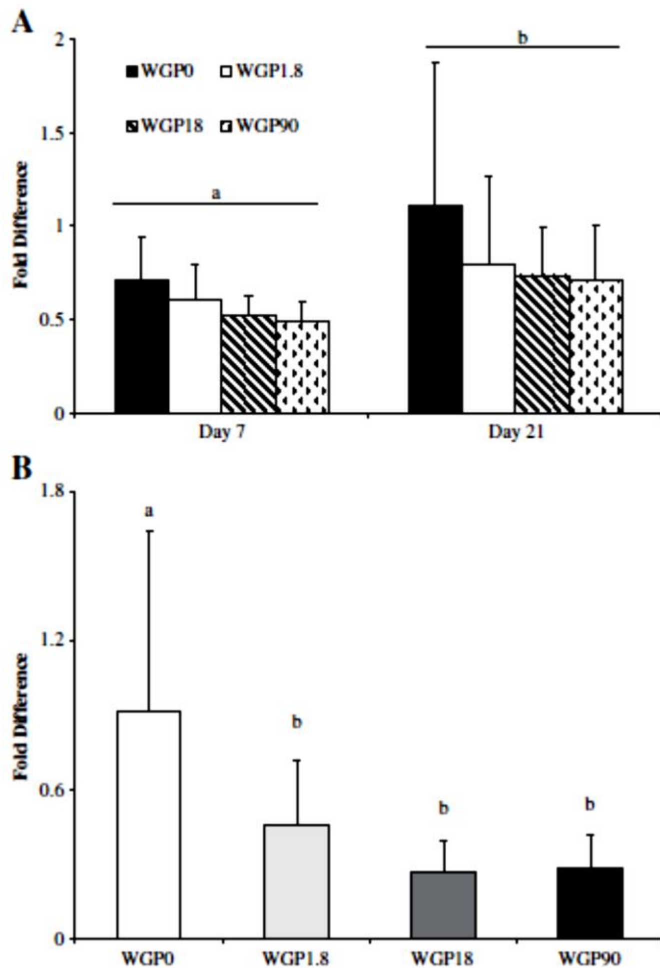

Fig. 2 – Transforming growth factor-β mRNA expression in the lung. A, TGF-β1 expression increased between days 7 and 21 (P = .01). Increasing dietary WGP tended to decrease TGF-β1 expression (P = .088). Model P = .06. B, TGF-β2 expression in the lung decreased with increasing dietary WGP (P = .01). Piglet age did not affect TGF-β2 expression (P = .49); therefore, data were pooled within diet. For WGP0, n = 15; for WGP1.8, n = 17; for WGP18, n = 15; and for WGP90, n = 16 samples. Data are expressed as fold-change overexpression in day 21 WGP0 animals and reported asmeans ± SD. Statistical significance was determined using the general linear model, Fisher least-significant-difference post hoc testing (SAS 9.2; P < .05). Different letter superscripts indicate significant differences. Numbers indicate dietary dose in milligrams per kilogram of BW per day.

2.10 Statistical Analysis

Analyses were performed using the PROC GLM (generalized linear model) procedure within SAS (Cary, NC). The initial model was day, diet, and day x diet. If diet was not significant, d7 and d21 data were combined. If day was not significant, data was combined within a diet group. Statistical significance was defined as $p \leq 0.05$, with trends reported when $p<0.10$. All data are expressed as means ± SD.

3. RESULTS

3.1 Formula intake and Body Weight

Formula intake and body weight were measured daily to determine if WGP affected acceptance of diet or growth. Formula intake and body weight were similar across all treatment groups. An average formula intake of 808 ± 167 ml/day was consumed for the first 7 days and 1488 ± 129 ml/day over the course of 21 days. The increase in body weight during the 7d or 21d period did not differ between groups. On average, piglets weighed 2.3 ± 0.5 kg and 4.5 ± 0.8 kg on d7 and d21, respectively. Therefore, inclusion of WGP had no effect on weight gain or dietary acceptance.

3.2 T Cell Populations

Flow cytometry was used to analyze T cell phenotypes in TLN (**Table 2**) and MSLN (**Table 3**). WGP supplementation did not affect T cell phenotypes. However, MSLN and TLN T helper cells decreased, while cytotoxic T cells increased between d7 and d21 ($p<0.05$), resulting in a decreased T helper/cytotoxic T cell ratio (**Figure 1**). There was a trend for dietary WGP to decrease the T helper/cytotoxic T cell ratio in the TLN ($p=0.0653$) with the ratio in WGP0 at 8.1 ± 3.4, WGP1.8 at 5.9 ± 1.9 , WGP18 at 5.3 ± 2.6 and WGP90 at 6.5 ± 1.7. Double positive T cells did not show the expected increase from d7 to d21 in either the MSLN or the TLN. In TLN, similar percentages of T cells were double positive on d7 and d21, 7.2 ± 0.02% and 9.1 ± 0.04%, respectively ($p=0.24$) (Table 2). In MSLN, when averaged over all diets, 7.1 ± 0.03% of T cells were double positive on d7, while 8.3 ± 0.03% of were double positive on d21 ($p=0.31$) (Table 3).

3.3 Tissue mRNA Abundance

The mRNA expression of various genes in tissue samples from the lung, TLN, and MSLN. Neither day nor diet altered lung dectin, IL-1α, IL-2, IL-4, IL-12, or TNF-α mRNA expression. However, dietary WGP supplementation tended to decrease TGF-β1 and decreased TGF-β2 (**Figure 2, Table 4**) mRNA expression in the lung. Although the effect of diet on lung TGF-β1 expression was minimal, lung TGFβ-1 mRNA expression significantly increased between d7 and d21 (**Figure 2A**, $p<0.05$). On the other hand, WGP affected lung TGF-β2 mRNA expression. On both d7 and d21, lung TGF-β2 mRNA expression was lower in all animals fed WGP compared to those fed formula alone

Table 4: Gene expression in Lung, TLNs and MSLNs of 7- and 21-day old Piglets consuming 0 to 90 WGP per kg BW per day

		Day 7				Day 21			
		WGP0	**WGP1.8**	**WGP18**	**WGP90**	**WGP0**	**WGP1.8**	**WGP18**	**WGP90**
Lung	**Dectin**	1.0±0.6	0.7±0.4	0.7±0.4	0.7±0.2	1.0±0.8	0.6±0.5	0.9±0.1	0.9±0.4
	TGF-β1[b]	0.7±0.2	0.6±0.2	0.5±0.1	0.5±0.1	1.1±0.8	0.7±0.3	0.7±0.3	0.7±0.3
	TGF-β2[c]	0.8±0.4	0.5±0.2	0.2±0.1	0.3±0.1	1.1±0.9	0.5±0.3	0.3±0.2	0.3±0.1
TLN	**Dectin**[b]	3.2±1.3	3.6±2.2	2.1±0.9	1.7±1.2	1.2±0.6	1.6±1.1	3.3±2.8	1.9±1.4
	TGF-β1	1.3±0.8	1.1±0.2	0.5±0.3	0.8±0.4	1.0±0.60	1.3±1.0	1.5±1.1	0.6±0.1
	TGF-β2	2.8±2.9	2.0±1.9	0.5±0.5	1.3±1.5	1.0±0.9	1.1±0.9	1.4±1.1	0.6±0.2
MSLN	**Dectin**	1.7±0.7	1.4±1.3	1.0±0.6	1.1±0.7	1.0±0.5	1.8±1.6	1.3±0.8	2.0±1.6
	TGF-β1	1.1±0.4	0.8±0.3	0.7±0.3	0.6±0.2	1.0±0.4	1.2±0.7	1.0±0.6	1.3±0.8
	TGF-β2[b]	0.9±0.2	0.7±0.1	3.2±5.0	0.6±0.2	0.8±0.7	1.7±0.7	1.1±0.7	1.5±1.0

Data are fold-change overexpression in day 21 WGP0 animals and reported as means ± SD.
[a] Numbers indicate dietary dose in milligrams per kilogram of BW per day.
[b] Expression of these genes in the indicated tissues differed between days 7 and 21 (general linear model, Fisher least-significant-difference post hoc testing [SAS 9.2]; P < .05).
[c] Lung TGF-β2 expression decreased with increasing WGP supplementation but was not affected by day (general linear model, Fisher least significant-difference post hoc testing [SAS 9.2]; P < .05).

(**Figure 2B**, p<0.01). IL-6 mRNA expression was not evaluated in the lung. MSLN mRNA expression of dectin, IL-2, IL-4, IL-6, IL-12, TFGβ-1 or TNF-α was not influenced by diet or day. However, MSLN TGFβ-2 mRNA expression increased from d7 to d21 (p<0.05) (**Figure 3,** Table 4). IL-1α mRNA expression could not be evaluated in MSLN because expression was below the detection limits of our assay. Neither day nor diet affected TLN IL-4, IL-6, IL-12, TGF-β1, TGF-β2 or TNF-α mRNA expression. Dectin mRNA expression was lower in TLN at d21 compared to d7 (p<0.05, **Figure 4,** Table 4). IL-2 and IL-1α were not analyzed in TLN because their expression was low in TLN. The single effect of dietary treatment with WGP was decreased TGF-β2 expression and a trend to decrease TGF-β1 expression in whole lung tissue. No consistent effects of dietary WGP treatment could be seen on tissue-wide mRNA expression of inflammatory (IL-2, IL-12, IL-6, IL-1α, TNF-α) or anti-inflammatory (IL-4, TGF-β1, TGF-β2) cytokines.

3.4 Mitogenic Cell Stimulation and Cell-mediated response

To determine whether cell proliferation was increased by dietary WGP, cells were stimulated with LPS or ConA. ConA stimulated, but not LPS stimulated, MSLN and TLN cells from all animals proliferated more

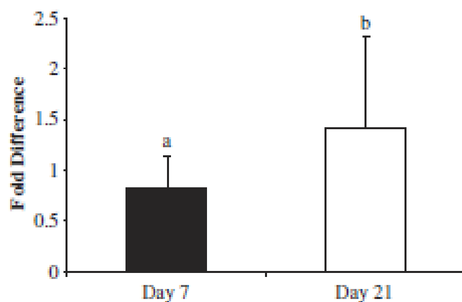

Fig. 3 – Transforming growth factor-β2 mRNA expression in MSLNs increased from days 7 to 21 in all pigs (model P = .02, day P = .02). No diet (P = .61) differences were detected; therefore, data were pooled within day. For day 7, n = 20 samples. For day 21, n = 43 samples. Data are expressed as fold-change overexpression in day 21 WGP0 animals and reported as means ± SD. Statistical significance was determined using the general linear model, Fisher's least-significant- difference post hoc testing (SAS 9.2; P < .05). Different letter superscripts indicate significant differences.

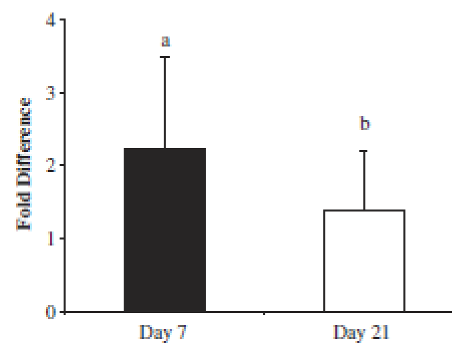

Fig. 4 – Dectin mRNA expression in TLNs was lower at day 21 compared with day 7 (model P = .02, day P = .004). No diet (P = .76) differences were detected; therefore, data were pooled within day. For day 7, n = 20 samples. For day 21, n = 43 samples. Data are expressed as fold-change overexpression in day 21 WGP0 animals and reported as means ± SD. Statistical significance was determined using the general linear model, Fisher least-significant-difference post hoc testing (SAS 9.2; P < .05). Different letter superscripts indicate significant differences.

Table 5. Mitogenic cell stimulation of TLN and MSLN cells isolated from 21-day-old animals

	ConA	LPS	BSA
MSLN	3.4±1.7[a]	1.9±1.4[b]	1.3±1.3[b]
TLN	4.2±0.85[a]	2.0±1.2[b]	1.3±1.1[b]

Data are expressed as log Δcpm and reported as means ± SD. Values within the same row with different letters are significantly different (general linear model, Fisher least-significant-difference post hoc testing [SAS 9.2]; $P < .05$). In both MSLN and TLN, cells stimulated with ConA proliferated significantly ($P < .05$) more than those treated with LPS or BSA. Dietary WGP did not enhance proliferation; therefore, data from all diet groups were pooled.

than cells stimulated with a protein control (BSA) ($p<0.05$, **Table 5**), however, there was no interaction between dietary WGP and stimulation in either MSLN or TLN cells.

3.5 Histomorphology and Immunohistochemistry

In order to evaluate the effects of WGP on morphology, lung sections were stained with H&E or anti-CD3 antibody. Lung sections had little T cell infiltration and no lesions (data not shown). Slides were analyzed by a board certified veterinary pathologist at the University of Illinois College of Veterinary Medicine who determined that there was no effect of WGP or day on $CD3^+$ T cell numbers or lung morphology (data not shown).

4. DISCUSSION

βGs are polysaccharides that occur as a principal component of cell walls and have been shown to affect a variety of biological responses. This study utilized a neonatal piglet model to further explore the effects of dietary WGP on lung immunity. Overall, lung immune development in neonatal piglets was minimally affected by treatment with dietary WGP at 1.8, 18 or 90 mg/kg BW/day. Additionally, WGP was found to have no negative impacts on growth, dietary acceptance or the immune characteristics analyzed.

Cytokines are one of several immunomodulatory factors that help protect neonates from pathogens. The cytokine TGF-β is of particular interest because of its immunoregulatory role during pregnancy and birth in humans as well as its role in the Th2 bias of neonatal immune responses [17]. In this study, TGF-β1 mRNA expression in the lung increased between d7 and d21 and TGF-β2 mRNA expression in the MSLN increased between d7 and d21. An increase in TGF-β1 likely contributes to a shift from a Th1 response to a Th2 response, which would be counter to the expected Th2 to Th1 shift seen with age. TGF-β1 down-regulates immune activation in intestinal epithelial cells and lamina propria immune cells [18], which may allow for initial colonization of the intestine [19]. In addition, TGF-β helps regulate innate IFN-γ production which is a signal that controls Th1 development [17]. Laouar and colleagues [17] demonstrated that blockage of TGF-β signaling in NK cells, the main source of innate IFN-γ production, increased NK cell number and therefore increased production of innate IFN-γ. All of this evidence suggests that the increase in TGF-β1 would decrease inflammatory conditions in the lungs and MSLN of d21 pigs. Interestingly, we found that WGP supplementation decreased TGF-β2 and tended to decrease TGF-β1 mRNA expression in the lung independently of age. This decrease in regulatory cytokine message is consistent with the role of WGP as an immune stimulator. However, this effect was not observed in either MSLN or TLN.

Typical developmental changes in T lymphocyte populations were not dramatically altered by dietary supplementation of WGP. The pig immune system is unique from humans because blood and lymphoid tissues express a significant number of double positive T cells. This subpopulation of T cells has been shown to increase with age and immunological experience [20]. Even though there was a trend for the population of double positive T cells to increase in both the MSLN and TLN, this increase did not reach significance. However, the size of this population in our animals was consistent with that published by others (between 8 and 64% of the circulating pool of T cells) [20-24]. Previous studies have shown a higher percentage of T helper cells than cytotoxic T cells in PBMCs of piglets (T helper/cytotoxic T cell ratio >1) until the 40th day of life [25]. T helper cells decrease after about one week and eventually become lower than cytotoxic T cells [25]. A similar shift in T cell populations in the MSLN and TLN was observed in the current study. In addition, the CD4/CD8 T cell ratio in TLN tended to be lower in pigs receiving any dietary WGP ($p<0.0886$). Thus, dietary WGP minimally affected the transition from the T helper cell predominance to an increased cytotoxic T cell presence, but had no effect on the timing of the shift.

Previous studies have examined the ability of βG to target sensitive immune cells through the oral route [26]. After absorption of the βG particles and uptake by the Peyer's Patches, the βG may remain in the gut-associated lymphoid tissue. However analysis of the T

cell populations of peripheral blood mononuclear cells and mesenteric lymph nodes isolated from the same piglets as studied herein demonstrated no effect of WGP on intestinal or immune development (16).

Previous studies examining the effect of dietary supplementation with βG on the response to systemic immunization or microbial challenge have produced mixed results. In one study, piglets vaccinated with atrophic rhinitis vaccine produced significantly less antibody when fed βG from *S.s cerevisiae* [27]. However, pigs injected with ovalbumin and receiving βG from *S. cerevisiae* at a dose of 0.005% mounted a higher antibody response [28]. Similarly dietary βGs from *S. cerevisiae* at a dose of 0.03% did not enhance the immune response to vaccination with porcine reproductive and respiratory virus 29,30]. Additionally, dietary WGP did not affect antibody or cell-mediated immune responses to influenza virus vaccination in mice [31]. However, piglets fed βG from *S. cerevisiae* and *Sclerotium rolfsii* for two weeks post-weaning were less susceptible to $F4^{+}$ ETEC infection [32]. Protection from $F4^{+}$ ETEC could not be repeated in gnotobiotic piglets fed βG from *S. cerevisiae* from 1 week of age [32]. In another study, WGP administered by subcutaneous injection increased survival rate, diminished bacterial load in the lungs and increased the proportion of bacteria-free mice after infection with anthrax [33]. Soluble βG from *S. cerevisiae* also had an anti-viral effect against porcine reproductive and respiratory virus in weanling pigs and was associated with an up-regulation of the Th1 cytokine IFN-gamma [34]. In contrast, more pigs fed 0.025% βG from *S. cerevisiae* died by d12 after *Streptococcus suis* challenge than control pigs despite increased average daily food intake [35]. It is unclear which factors affected the outcomes of these studies. However, it is clear that βG have mixed effects on immune responses. Generally, they appear to protect against microbial challenges. Based on the results of our study, this may be due to decreases in anti-inflammatory cytokines like TGF-β.

Toll Like Receptors (TLRs) and other innate receptors were not examined in this study. However, dectin-1, a PRR for βG, expression was measured. In part, dectin was chosen because the mechanism by which this βG receptor translates recognition into intracellular signaling, stimulates cellular responses, and participates in coordinating the adaptive immune response is well-characterized [36]. We found that dectin-1 mRNA expression in the TLN decreased from from d7 to d21. Surprisingly, dectin-1 mRNA expression in MSLN, TLN and lung was not affected by dietary WGP supplementation. Dectin-1 is expressed mainly on phagocytes, especially macrophages and dendritic cells. It has been suggested that dectin-1 signals alone are sufficient to trigger phagocytosis [37] and that dectin-1 signaling in combination with TLR signaling can regulate cytokine production [38]. The decrease in TLN dectin-1 mRNA seen in this study may be associated with altered phagocytosis and cytokine production in the TLN. However, we did not measure phagocytic activity, and cytokine mRNA expression in the TLN was not affected by piglet age.

Evidence suggests that the biological activity of βGs is associated with their structure which, in turn, is dependent on the source and method of extraction and isolation. Processing [39-41], solubility, degree of polymerization (solubility increases as degree of polymerization increases) [40,41], size (high molecular weight (100-200 kDa) fractions being most active while fractions from the same source with lower molecular weights have no activity [42-44]), charge of polymers, and structure also contribute to functionality of βG. Several previous studies used Hunter and co-workers' [45] method of extraction with some modifications [29] with positive biological results. In our study, the βG was extracted from *S. cerevisiae* using a proprietary technology. This method produces a whole glucan particle in which the outer surface of mannoprotein and inner cellular contents are removed to expose the β-(1,3/1,6)-glucan. Perhaps this additional processing decreased recognition of the particle and therefore altered the absorption of the molecule or the ability of the molecule to be recognized by PRRs. This processing also may have decreased the solubility of the particle leading to limited absorption or decreased distribution of the βG in the formula due to the feeding method that was utilized.

In conclusion, the effects of WGP on lung immunity in the healthy neonatal piglet were minimal. Dietary WGP reduced TGFβ mRNA expression in lung and tended to decrease the T helper to cytotoxic T cell ratio in the TLN. Further research, targeted to examine effects on innate immune mechanisms or acute microbial challenges, is needed to increase our understanding of the ability of WGP to enhance the biological activity of the neonate's immune system.

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