13 NONCLINICAL TOXICOLOGY

13.2 Animal Toxicology and/or Pharmacology

Nonclinical studies of bovine-derived plasma proteins containing immunoglobulins consistently demonstrate positive effects across multiple species on growth, food intake, and nutritional status in animal populations with GI inflammation. A number of nonclinical studies evaluating the effects of immunoglobulins on the intestinal mucosa in rats and mice, and on intestinal inflammation in piglets, are available in the literature. These studies have evaluated the impact of plasma proteins within ENTERAGAM on lean body mass and growth, intestinal barrier function, and cytokine and immune response. A number of nonclinical studies have also demonstrated that the plasma proteins similar to those found in ENTERAGAM improve lean body mass and growth through increased protein utilization.

Table 1. Lean Muscle Mass and Growth Following Dietary Management with Plasma Proteins

Species	**Model/Indication**	• **Impact of Dietary Management with Plasma Proteins**
Piglets	Early Weaned (21 d), N=48 (4 groups), 6% PP or FP w/ or w/o antimicrobial medication diets for 15d	• Increased average daily weight gain ($p = 0.04$) and average daily food intake ($p < 0.01$). The addition of antibiotics induced a further increase in average daily food intake. • These results indicated that feeding early-weaned piglets with PP improved growth performance.
Piglets	Early Weaned (14 d), N=24 Pair-fed for 24 d, Control (extruded soy protein) or 10% PP diet	• Significantly increased mean daily body weight gains (+23%; $p < 0.05$) and food conversion efficiencies (expressed as the ratio of weight gain to protein intake) (19%; $p < 0.05$). The greatest differences in weight gain (45%) and food conversion efficiency (43%) observed between 0 and day 8. • Significantly greater lean body mass (~16%; $p < 0.05$). • No effect on mass of the small intestine, stomach, liver, or spleen. • Significantly lower circulating urea concentrations (40%; $p < 0.05$), indicating greater retention of nitrogen and reduced amino acid catabolism. • Increased total BMC (29%, $p < 0.05$), bone mineral density (15%, $p < 0.05$), and BMC per kg (11%, $p < 0.05$). • These results demonstrate that feeding early-weaned pigs diets containing PP rather than extruded soy protein increased the efficiency of dietary protein use for weight gain and lean tissue growth, and that this response is mediated in part by decreased amino acid catabolism.
Piglets	Early Weaned (14 d), N=96 Fed 16 d, 3 dietary groups: Control, PP, or PP-PF/C	• Significantly increased weight gain (43%; $p < 0.05$). • Significantly increased protein intake (33%; $p < 0.05$). • Significantly increased the efficiency of dietary protein conversion efficiencies (~18%; $p < 0.05$). • No effect on crypt cell proliferation, crypt depth or villous height in the jejunum or ileum. • Intravillous lamina propria cell density in the jejunum was lower ($p < 0.05$). • Plasma urea concentrations were also 40-42% lower ($p < 0.05$). • These results indicate that feeding weaning pigs diets containing PP enhanced dietary protein conversion efficiency, reduced intestinal mass and the density of intravillous lamina propria cells and reduced the circulating urea concentration. Feeding PP also increased the efficiency of dietary protein utilization, in part, by decreasing amino acid metabolism.

Piglets	Early Weaned (14 or 21 d) N=502; (5 experiments) Control diet, 8% PP, SBI and/or porcine Ig fraction in diet to equal 50, 100 or 150% IgG level of PP diet	• Growth rate and feed intake increased in the critical post weaning period (0-7 days) with the 8% PP diet, SBI and porcine Ig fraction diets compared to control ($p < 0.01$). Diets containing SBI or the porcine Ig fraction resulted in similar or greater improvements in growth rate and feed intake compared to the 8% PP diets. • The IgG component of porcine or bovine plasma stimulates equal or better growth performance, indicators of nutritional status, compared to whole plasma products. In addition, the early growth responses are more consistently maintained into later postweaning periods.
Mice	Weaned (21 d), N=180, Control diet or control diet +4% PP, +8% PP or 12% PP	• Significantly increased ADG ($p < 0.01$). • Significantly increased ADFI ($p < 0.01$). • Significantly increased G/F, with a more consistent and pronounced effect in males ($p < 0.05$ to $p < 0.01$). • The results indicate mice respond to dietary intake of PP with increases in ADFI, ADG and G/F. 8% PP was the optimal concentration used. There was no significant difference; the PP ranged from 4-12%.

ADFI = average daily food intake; ADG = average daily gain; BMC = body bone mineral content; FP = fish proteins; G/F = gain to feed ratio; Ig = Immunoglobulins; PP = plasma proteins; PP-PF/C = plasma protein, pair fed to control

Dysregulated cytokine production associated with intestinal inflammation can promote an increase in enteric epithelial tight junction permeability with resultant antigenic penetration of the gut barrier. These effects may be ameliorated through SBI's prevention of pro-inflammatory cytokine expression, including TNFα, INFγ, and interleukins (*e.g.*, IL-1, IL-6, IL-8, IL-17), thus facilitating maintenance of normal GI function and nutritional utilization. Nonclinical studies have demonstrated that dietary inclusion of plasma proteins improves intestinal barrier dysfunction damaged by inflammation. Plasma proteins within SBI prevent the release of mucosal cytokines in intestinal inflammation in animals and alter the lymphocyte response to immune activation (Table 2).

Table 2. Inflammatory and Immune Response Following Dietary Management with Plasma Proteins

Species	Model/ Indication	Impact of Dietary Management with Plasma Proteins
Pig	*E. coli* (ETEC K88) Infection, N=48 (4 groups) 6% PP or FP (control) w/ (M) or w/o (NM) antimicrobial medication diets for 15 d	• Saliva IgA concentrations were reduced in the PP group compared to the FP-NM group; ICI histological scores were similar across treatment groups. • Expressions of TNFα and IL-8 were lower in pigs fed PP than those fed FP, regardless of exposure to ETEC K88 ($p < 0.01$). • Expression of INFγ was lowered by PP and a medication interaction was noted. • These results indicated that PP feeding to early-weaned piglets challenged with ETEC K88 improved growth performance, reduced inflammation in animals, reduced IgA secretion, decreased intestinal mucosal damage, and reduced pro-inflammatory cytokine expression within the intestine.

Pig	Post-weaning period, Weaning (17-19 d), N=48 Control, 2.5% or 5.0% PP	• 2.5% and 5% PP diets reduced colonic paracellular permeability ^{14}C-inulin *vs* control on day 7 post-weaning. Both 2.5% and 5% PP diets reduced ileal ^{3}H-mannitol and ^{14}C-inulin permeability on day 14 post-weaning. • 2.5% and 5% PP diet reduced colonic short-circuit current (an index of net electrogenic ion transport) at days 7 and 14 post-weaning *vs* controls in ileum ($p < 0.05$). 5% PP fecal scores were reduced at day 14 ($p < 0.05$). • Histological analysis revealed fewer lamina propria cells in ileum and colon from pigs fed 2.5 and 5% PP at days 7 and 14 post-weaning. • Levels of TNFα were reduced in the colon but not ileum from pigs fed 5% PP at day 7 and 14 post-weaning *vs* controls ($p < 0.05$). IFNγ levels were lower *vs* controls in both PP groups in the ileum and colon at day 7 but not day 14 post-weaning. • These results demonstrate that dietary inclusion of PP had beneficial effects on the intestinal barrier function, inflammation and diarrhea in weaned pigs.
Rat	SEB Weaned (21 d), N=40 (4 groups) Fed treatment diets for up to14 days; Control, SEB, SEB-8% PP SEB-2% IC	• SEB-increased the water content of feces, which was prevented by diets containing either PP ($p < 0.002$) or IC ($p < 0.001$). • In Peyer's patches, PP reduced the SEB-induced increase in T lymphocytes ($p < 0.10$) as well as the percentage of activated T helper cells ($p < 0.05$). • The effects of PP augmentation on the lymphocyte populations of the GALT in rats challenged with SEB support the view that PP can modulate the immune response.
		• SEB increased lymphocyte populations of T-γδ cells by 38%; ($p < 0.001$), natural killer cells by 59% ($p < 0.05$) and the number of activated T lymphocytes by 148% ($p < 0.001$) in lamina propria. • PP and IC decreased the effects of SEB on the above lymphocyte subsets ($p < 0.05$). SEB increase intraepithelial activated lymphocytes by 117%; this was reduced by consumption of PP ($p < 0.01$). • The effects of PP and IC on intestinal lymphocyte subsets suggest that oral PP can modulate the degree of activation within the GALT.
		• PP prevented the SEB-induced increase in IFNγ, IL-6, and LTB_4 in Peyer's patches and in the mucosa ($p < 0.05$). • PP increased IL-10 and mature TGFβ concentrations in intestinal mucosa. • PP and IC increased the mature:total TGFβ ratio ($p < 0.05$). • The preventive effects of PP and IC on intestinal inflammation involve modulation of intestinal cytokines, which is characterized by an increased expression of anti-inflammatory cytokines.

Mouse	2% DSS-induced IBD model Diets contained either 0, 11.25, 22.5 g/kg of SBI for 7 or 14 d	• After 14 days the lower doses of SBI reduced IL-1α, IL-1β, IL-4, IL-6, IL-10, G-CSF, MCP-1 and KC, SBI reduced cytokine induction in response to DSS in mice and may have value in human therapy.
	KO model of spontaneous colitis Weaning (19 d) Fed 37 d Diets contained either PP 8%, SBI or milk proteins.	• The colitis syndrome of the KO mice was characterized by a 13-fold increase in recruited lymphocytes ($p < 0.05$) in the lamina propria, mainly the Th subset (50.2 ± 2.8% in KO *vs* 34.2 ± 1.4% in WT mice; $p < 0.05$). • The percentage of activated Th lymphocytes was also higher in KO (17.0 ± 0.5%) than in WT mice (8.7 ± 0.9%; $p < 0.05$). These effects were reduced by PP ($p< 0.05$). • PP and IC reduced 20-50% the colonic concentration of IL-2, IL-17, MCP-1 and MIP-1β (all $p < 0.05$) while the effects on TNFα, IFNγ and iNOS were lower (10-15% reduction; $p < 0.05$). • Both PP and IC increased the percentage of regulatory Th lymphocytes in the lamina propria ($p < 0.05$). • The effects of PP and IC on reducing the expression of colitis markers in the KO mouse model involves modulation of mucosal colonic cytokines and lymphocyte recruitment.
Mouse	Acute Lung Inflammation. Weaned (19 d), N=42-48 (6 groups) Fed treatment diets for up to 14 days; Control Control-8% PP Control-2% IC, LPS LPS-8% PP LPS-2% IC	• PP and IC attenuated the 35-fold increase in leucocytes in BALF which followed intranasal LPS challenge. • PP attenuated increased activated monocytes in BALF following LPS challenge. • PP prevented the increased infiltrated leukocytes in lung tissue following LPS challenge. • In unchallenged mice, both PP and IC reduced the percentage of resident neutrophils and monocytes ($p < 0.05$). • PP and IC prevented LPS-dependent monocyte activation in the blood (CD14+; $p < 0.05$). • At 6 hours, the LPS-induced pro-inflammatory cytokines (IL-1α, IL-1β, IL-6, G-CSF, and TNFα) were reduced by both PP and IC. • IL-10 production was increased in the LPS-PP and LPS-IC treatment groups compared to LPS treatment alone. • Both PP and IC eliminated the LPS increased iNOS expression in the lung tissue. • For chemokines, PP reduced LPS-induced CXCL1 and CCL3 expression at 24 hours and significantly reduced CCL2, CCL3 and CCL4 production at 6 hours ($p < 0.05$). • Dietary PP or IC interacts with the immune cells of GALT and reduces the pulmonary response to an LPS challenge. These results are consistent with dietary management of lung inflammation.

BALF = bronchoalveolar lavage fluid; CCL = Chemokine (C-C motif) ligand; CD14+ = cluster of differentiation antigen 14; CXCL1 = Chemokine (C-X-C motif) ligand 1; DSS = dextran sodium sulfate; ETEC K88 = enterotoxigenic *Escherichia coli*, K88 strain; FP = fish protein; G-CSF = Granulocyte colony-stimulating factor; HIV = human immunodeficiency virus; IBD = inflammatory bowel disease; IC = immunoglobulin concentrates; IFNγ = interferon-γ; IL-1α = interleukin-1α; IL-4 = interleukin-4;

IL-6 = interleukin-6; IL-8 = interleukin-8; IL-10 = interleukin-10; IL-17 = interleukin 17; iNOS = inducible nitric oxide synthase; KC = keratinocyte-derived cytokine; KO = knock out; LPS = lipopolysaccharide; LTB_4 = leukotriene B4; MCP-1 = monocyte chemotactic protein 1; MIP-1b = macrophage inflammatory protein; PBMC = peripheral blood mononuclear cell; SBI = serum-derived bovine immunoglobulin/protein isolate; PP = spray-dried plasma protein; SEB = *Staphylococcus aureus* enterotoxin B; TGFβ = transforming growth factor β; Th = T helper; TNFα = tumor necrosis factor α; WT = Wild Type

These data support the hypothesis that a diet containing plasma proteins prevents alterations in epithelium structure during inflammation, thereby reducing intestinal permeability, increasing absorption, and improving intestinal barrier function and the modulation of the immune response by limiting immune activation.