IMMUNOPROTECTIVE EFFECTS OF LIPOPOLYSACCHARIDE DETOXIFICATION BY A NOVEL MICROBIAL-DERIVED ALKALINE PHOSPHATASE

BY

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THESIS

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Abstract

Lipopolysaccharide (LPS) is a major outer cell wall component of Gram-negative bacteria, including *Escherichia coli* and *Salmonella enterica,* which have important health and economic consequences in animal agriculture. LPS is a recognized endotoxin that stimulates toll-like receptors, which are present on cells that contribute to inflammatory responses and, via downstream signaling pathways, results in the release of pro- and anti-inflammatory mediators to assist with pathogen clearance and tissue repair.

During weaning, piglets are subjected to numerous psychological and environmental stressors including mixing, lower ambient temperature and change in diet. Due to the piglets' immature digestive and immune system, weaning is associated with growth plateaus, increased incidence of diarrhea, increased bacterial translocation and endotoxin load, and increased mortality. Increased concentrations of LPS in circulation perpetuate post-wean syndrome and drive further gastrointestinal tract (GIT) morphological and digestive perturbations. Weaning is also characterized by reductions in intestinal alkaline phosphatase (IAP), which has biological roles including detoxifying LPS in the intestinal lumen and maintaining an alkalotic pH to modulate gut microbiota. IAP is a homodimeric enzyme that catalyzes the release of an inorganic phosphate from the lipid A moiety of LPS, resulting in the considerably less immunostimulatory product, monophosphoryl lipid A (MPLA).

Based on IAP's ability to reduce the inflammatory effects of LPS in the GIT and the difficulty in creating a viable commercial product from a mammalian-derived enzyme; a microbe was engineered to produce an alkaline phosphatase (MAP). However, the ability of MAP to detoxify LPS *in vitro* and *in vivo* has not been investigated. In chapter 2, to test the hypothesis that MAP would detoxify LPS similarly to IAP, RAW264 macrophages and primary porcine alveolar macrophages were treated with vehicle, IAP, MAP, *E. coli* or *S. enterica* LPS, LPS that

had been pre-incubated with MAP (MPLA), or LPS that had been pre-incubated with IAP (iMPLA). In support of the hypothesis, cells treated with MPLA had significantly lower TNFα, IL-6, IL-1β, and IL-10 gene expression as compared to cells treated with LPS alone. Furthermore, novel findings indicate an increased efficacy for MAP to detoxify LPS as compared to IAP. MPLA treated cells had significantly lower levels of pro- and anti-inflammatory cytokine production as compared to iMPLA-treated cells. We then determined the serum cytokine profiles of piglets 2 days post-wean after being challenged with saline, MPLA, or LPS for four hours. Piglets i.p. injected with MPLA were more active and ate more over the time period than LPS injected piglets. Furthermore, MPLA piglets had altered serum cytokine profiles indicating an ameliorated immune response to the challenge.

In chapter 3 we investigated the effects of exogenous MAP supplementation on post-wean syndrome in piglets on a standard phase 2 diet. We found that adding 4,000 IU/kg body weight to a phase 2 diet for two weeks post-weaning resulted in a higher average daily gain and body weight. MAP-fed piglets had increased villus height and decreased crypt depth, and an increased villus height to crypt depth ratio in the duodenum, jejunum, and ileum. Furthermore, MAP-fed piglets were protected against weaning-induced downregulation of tight junction protein ZO-1 and inflammation-induced increases in claudin-1. IAP is known to be downregulated post-weaning due to increased inflammatory mediators which inhibit gene expression. Therefore, we sought to determine the effects of exogenous MAP supplementation on endogenous IAP gene expression and found that dietary MAP-supplementation significantly increased IAP gene expression in the duodenum, jejunum, and ileum. Furthermore, alkaline phosphatase activity was significantly higher in the digesta and mucosa of the duodenum, jejunum, and ileum of weaned pigs eating a MAP-supplemented diet.

This is the first study, to our knowledge, to characterize the effects of exogenous alkaline phosphatase supplementation on post wean syndrome in piglets. Our data indicates that MAP may serve as a potential dietary additive to mitigate the effects of LPS- and weaning-induced inflammation on innate immune cell cytokine production, GIT permeability and morphology, tight junction protein perturbations, and downregulation of IAP gene expression and activity.

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LIST OF ABBREVIATIONS

IAP	Intestinal alkaline phosphatase
iMPLA	IAP-detoxified LPS
LPS	Lipopolysaccharide
MAP	Microbe-derived alkaline phosphatase
MPLA	MAP-detoxified LPS

CHAPTER 1: LITERATURE REVIEW

Post-Wean Syndrome

In the modern swine industry, weaning is the most challenging and stressful point in the pig's life due to major nutritional, psychological, and environmental stressors. The piglet is removed from sow's milk and transferred to a novel environment with different ambient temperature and mixed with other piglets. The post-weaning phase is often accompanied by reduced growth performance, reduced feed intake, and increased incidence of diarrhea and bacterial translocation[1,2]. Commercial pigs are typically weaned between twenty-one and twenty-eight days of age and have immature digestive and immune systems, which contribute to the pathology of the post-weaning syndrome.

The immature digestive system of the piglet and decreased feed intake due to post-weaning syndrome are associated with significant gastrointestinal tract (GIT) disturbances characterized by changes in intestinal morphology that increase susceptibility to diarrhea and growth plateaus[3,4]. The epithelial lining of the small intestine is covered in villi, small finger-like projections, to increase surface area for digestion and absorption[5]. Optimal function of the small intestine favors long villi. At the base of the villi, the mucosal surface of the small intestine has tubular glands that open into the intestinal lumen known as crypts. Crypts contain stem cells required for replacement of the epithelial cell lining[6]. There is a brief period of villous atrophy and crypt hyperplasia following weaning that leads to impaired gut absorptive capacity and decreased digestive and brush-border enzymatic activity[3,7,8]. The large intestine is composed of the caecum, colon, and rectum, and the main functions are fluid and electrolyte absorption and the supply of a physical barrier against microbial translocation. Weaning induces a decreased crypt density in the caecum of the large intestine and a transient reduction in the absorptive

capacity of the colon[4,9]. Stress from a novel environment and littermates also contribute to decreased villus height and increased crypt depth in the weaned piglet. Glucagon, a stress-associated catabolic hormone that mobilizes stored energy, had an elevated blood concentration in piglets' day two to five post-wean and may contribute to intestinal changes[10]. Other stress hormones, cortisol, and corticotrophin-releasing factor, also had elevated blood plasma concentration after weaning[11,12].

Post-weaning diarrhea (PWD) is part of the post-weaning syndrome and represents one of the most significant economic wastes for the pig industry. PWD is characterized by frequent discharge of watery feces during the first two weeks post-weaning. One of the essential functions of the small intestine is nutrient digestion and absorption. This includes secretion of fluids and electrolytes from crypt cells and nutrient absorption via enterocytes from the intestinal brush-border[13,14]. Weaning reduces the small intestines' capacity for net absorption of fluid and electrolytes and leads to malabsorption of nutrients[15,16]. Following weaning, a net secretory condition can occur when the fluid and electrolyte influx into the GIT lumen exceeds the efflux into the blood, and this contributes to the pathology of PWD[13,14]. PWD is also associated with increased fecal shedding of a significant number of enterotoxigenic *E.coli* serotypes that proliferate in the small intestine[17,18].

Another significant role of the small intestine is to act as a barrier against antigens and pathogens. The gastrointestinal tract is lined with a single layer of epithelial cells that form a selective barrier and act as the first line of defense against potentially harmful compounds and microorganisms in the intestinal lumen. Intestinal barrier dysfunction is characterized by increased intestinal permeability, or "leaky gut," which allows harmful immunogenic agents to cross the epithelium and gain access to protected tissues and systemic circulation. This breach of

the epithelial lining and subsequent translocation of luminal contents leads to increased inflammation, malabsorption, diarrhea, and potential enteric disease [4,19,20]. The various environmental and psychological stressors on the weanling piglet contribute to the deterioration of the small intestinal barrier function, most likely through the release of stress mediators, including the aforementioned corticotrophin-releasing factor and adrenal glucocorticoids[21–23]. Morphological changes may impair intestinal barrier function and lead to increased gut permeability, which results in increased bacterial translocation and inflammatory response. Enterocytes are joined together by tight junctions that consist of proteins which function to connect the cytoskeletons of adjacent enterocytes[24]. Tight junctions are mainly constructed from the transmembrane protein complexes occludins and claudins, and the cytosolic protein zonula occludens. Alterations in tight junction proteins are biomarkers of increased intestinal permeability. Increased translocation of enteric pathogens, including endotoxins from Gram-negative bacteria, disrupt these proteins and increase intestinal permeability[25]. The immature nature of the weanling piglets immune and digestive systems contributes to the duration and severity of GIT dysfunction.

Impaired barrier function led to increased pathogenic bacterial load and decreased immune competence by decreasing cell numbers or increasing immunosuppressive mechanisms[26]. Increased bacterial load could lead to excessive immune responses that are deleterious to the host, and an increase in immunosuppressive mechanisms to protect against serious damage from the aberrant immune response. However, immunosuppression can slow pathogen clearance and facilitate disease states. Nursery piglets are immuno-deficient and rely on sow's milk for growth, survival, and immunity. Immunoglobulin A (IgA) is an antibody that plays an essential role in the immune function of mucous membranes and removal of IgA and other bioactive compounds

derived from sow milk contribute to the weaned piglets susceptibility to disease[27–29]. The immaturity of the intestinal immune system reduces the ability of the weaned pig to mount an appropriate immune response to pathogens and its ability to tolerate dietary antigens[29,30].

LPS, TLR4, and Immune Signaling Pathways

The immune system is divided into the innate and adaptive immune systems. The innate immune system is a generalized but swift response to pathogens and consists of skin, mucous membranes, and defense leukocytes. The innate immune system also includes antigen presenting cells (APC), such as macrophages and dendritic cells, that ingest antigens and pathogens, digest them, and display part of the ingested material on their cell surface in the context of a major histocompatibility complex (MHC). The MHC is a group of genes that code for cell surface proteins that allow the immune system to recognize self versus nonself peptides and appropriately bind the correct effector immune cells.

The adaptive immune system takes over if the innate response is unsuccessful, and the slower system is highly specialized. The adaptive immune system can remember specific antigens and combat them more efficiently and effectively during future encounters. It is made up of B and T lymphocytes and antibodies. When gut integrity has been compromised from various stressors, the goal of the innate response is to rapidly initiate effector functions to prevent extensive inflammation and sepsis[31]. The innate immune system plays a more significant role than the first line of defense by telling antigen presenting cells to activate and differentiate T cells towards the appropriate effector phenotype. T cells are responsible for the cell-mediated response of adaptive immunity. The type of T cell that the progenitor will polarize into is dependent upon the pattern of cytokine secretion from the APC. There are two classes of the major histocompatibility

complex; MHC I presents endogenous antigens to cytotoxic T cells ($CD8^+$), and MHC II presents exogenous antigens to helper T cells ($CD4^+$). $CD8^+$ T cells do not continue to differentiate and are destined to be cytotoxic, but $CD4^+$ T cells have five main types (T_H1, T_H2, T_H17, T_{FH} and T_{reg}) that promote distinct responses to different types of infections.

T_H1 cells control intracellular bacteria that infect immune cells by inducing the infected immune cell to self-destruct. T_H2 cells specialize in promoting responses to parasitic infections while T_H17 T cells secrete specific cytokines to recruit neutrophils for clearance of extracellular pathogens. T_{FH} T cells are the most recently discovered subset of $CD4^+$ cells and have been implicated in signaling to B cells. T_{FH} cells are critical for protective immune responses and are necessary for the development of most high affinity antibodies and memory B cells. Finally, T_{reg} or regulatory T cells are responsible for maintaining self-tolerance and suppressing T_H cells when their activities become detrimental. When activated, T_H1 and cytotoxic T cells release cytokines to help regulate the immune response and activate other cells. Interferon-γ is one such cytokine released and is an activator of macrophages and an inducer of MHC II expression. B cells provide the humoral immune response and develop in the bone marrow. They are characterized by cell membrane antigen receptors made from antibody molecules. Following maturation, the naïve B cells migrate to secondary lymphoid organs to interact with other components of the immune system. Antigen binding and co-stimulation from helper T cells activate B cells to proliferate and differentiate in memory B cells or plasma B cells that secrete antibodies. Memory cells form after the initial infection and are required for a more robust immune response following secondary infection. Effector B cells secrete antibodies with the same specialized antigen binding site as their plasma membrane-bound form and are used by the immune system to identify foreign objects. There are five classes of antibodies (IgA, IgD, IgE,

IgG, and IgM), and they work directly or indirectly on pathogens. Directly, antibodies work through agglutination, precipitation, neutralization, or direct lysis of pathogens. Indirectly, they activate the complement system, which activates macrophages through opsonization, works as a chemoattractant for other immune cells, and contributes to the lysis, agglutination, and neutralization of pathogens and antigens. Antibody function provides the groundwork for vaccine development.

The immune systems have evolved to recognize specific patterns, and the innate immune response is initiated when homeostatic functions are perturbed and surveilling immune cells recognize these patterns. Danger associated molecular patterns (DAMP) and pathogen associated molecular patterns (PAMP) are two such patterns that are recognized. DAMP are endogenous cell-derived molecules in response to tissue damage from stress such as necrosis, trauma, or cancer. PAMP are present in a diverse amount of microorganisms and are necessary for the organism to function, but are absent in the host to promote immunity[32,33]. PAMP are typically microbial nucleic acids, lipoproteins, surface glycoproteins, or membrane components. PAMP and DAMP bind to specific receptors that influence gene expression and regulate the immune functions of clearance, autophagy, phagocytosis, and recruitment of other immune cells through downstream signaling molecules.

Toll-like receptors (TLR) are transmembrane proteins found on cells that contribute to inflammatory responses, such as macrophages or epithelial cells of mucous membranes, which recognize PAMP and DAMP and mediate inflammatory effects through downstream signaling pathways. TLR have a leucine-rich repeat region and a cytoplasmic tail which has a Toll/interleukin-1 (IL-1) receptor (TIR) domain. While TLR are prominently expressed on

antigen presenting cells, other cells, including T cells, have been recently shown to express particular types of TLR[34].

TLR4 recognizes lipopolysaccharide (LPS), a primary cell wall component of Gram-negative bacteria that can contribute to the pathology of post wean syndrome, such as *Escherichia coli* and *Salmonella.* The bacterial cell wall component spontaneously releases into the host circulation, where it interacts with several proteins (TLR4, MD2, CD14, LBP) and initiates the innate immune system inflammatory response[35,36]. LPS is structurally organized into an outer polysaccharide region containing the O antigen; and an inner lipid moiety containing lipid A. Specifically, TLR4 recognize the lipid A region of LPS as a PAMP[37].

LPS-binding protein (LBP) is a glycosylated acute phase protein in which production in the liver is upregulated during the acute phase of a Gram-negative bacterial infection[38]. LBP exclusively binds the lipid A moiety of LPS with high affinity[39,40]. Once the LBP-LPS complex forms, myeloid-origin glycoprotein CD14 binds [41]. CD14 is available attached to the cell membrane via a glycosylphosphatidylinositol anchor (mCD14) or as a soluble form (sCD14) released from cells. sCD14 can bind LPS directly without LBP, and then the sCD14-LPS complex can activate cells that are lacking mCD14, such as endothelial cells and fibroblasts[42]. CD14 requires a receptor complex, TLR4/MD2, to initiate signal transduction and downstream inflammatory mediators. TLR4 associates with soluble glycoprotein MD2, which is required for TLR4 activation and LPS-sensing[43]. LPS, in conjunction with LBP and CD14, is delivered to TLR4/MD2 on the cell membrane, and the lipid A moiety is recognized by MD2 and results in signal transduction cascades within the cell[44].

The MyD88-dependent and independent pathways initiate a pro-inflammatory response mediated by nuclear factor (NF)-κB binding to promoters that regulate inflammatory genes.

Following the MyD88-dependent pathway, after ligand binding the TLR/IL-1R dimerize and undergo a required conformational change to recruit downstream signaling molecules including adaptor molecule myeloid differentiation primary-response protein 88 (MyD88), IL-1R-associated kinases (IRAK), transforming growth factor-β (TGF-β)-activated kinase (TAK1), TAK1-binding protein 1 (TAB1), TAB2 and tumor-necrosis factor (TNF)-receptor-associated factor 6 (TRAF6)[33,45]. The conformational change of TLR/IL-1R recruits MyD88 to the cytoplasmic TIR domain, where it facilitates the interaction of IRAK4 with the receptor complex. Following this binding, IRAK4-mediated phosphorylation of specific residues in the activation loop of IRAK1 initiates the kinase activity of IRAK1. The activated IRAK1 auto-phosphorylates, thereby allowing TRAF6 to bind to the structure, the IRAK1-TRAF6 complex leaves the receptor and interacts at the plasma membrane with another preformed complex consisting of TAK1, TAB1, and TAB2 or TAB3. The interaction between the two complexes induces phosphorylation of TAK1 and TAB2 or 3, which will then translocate with TRAF6 and TAB1 to the cytoplasm of the cell. TAK1 is activated in the cytoplasm, and in turn activates IκB kinases (IKK), which phosphorylate IκB, and lead to their degradation and the ultimate release of the transcription factor NF-κB and subsequent cytokine production. Conversely, in the MyD88-independent pathway, TLR3 and TLR4 activate TRIF-dependent signaling, which activates NF-κB and IRF3, resulting in the induction of pro-inflammatory cytokines and type 1 interferon.

LPS-induced cellular response

Once NF-κB has translocated to the nucleus, inflammatory mediators like cytokines and chemokines are differentially activated based on the cell type. The innate immune response results in a rapid burst of pro-inflammatory cytokines into the surrounding tissue and circulation,

and recruits effector cells, such as neutrophils, to travel to the site of infection for assistance in the clearance of the pathogen. The major secreted pro-inflammatory cytokines following an LPS challenge include tumor necrosis factor-α (TNFα), interleukin (IL)-1β, and IL-6. TNFα and IL-1β share several biological roles, such as activating neutrophils, and high concentrations in the circulation of these two cytokines can result in numerous pathophysiological effects such as fever and multiple organ failure[46–48]. IL-6, while still pro-inflammatory, is considered to be less potent than TNFα or IL-1β, and can be induced by those two cytokines. IL-6 can also be anti-inflammatory by inhibiting the synthesis of TNFα and IL-1β and inducing the release of soluble TNF receptors[49]. IL-4 and IL-10 are two major anti-inflammatory cytokines released following TLR4 stimulation. IL-4, mainly synthesized by activated T-lymphocytes, has biological roles in B cell proliferation and inhibition of inflammatory cytokine production[50,51]. IL-10 diminishes excessive inflammatory reactions, reduces antigen presentation, and inhibits T cell activation[52]. Generally secreted from mononuclear phagocytes, endothelial and epithelial cells, IL-8 is a key mediator in the inflammatory response[53]. A product and inducer of NF-κB, IL-8 secretion is also induced by LPS, TNFα, and IL-1β. In the extracellular environment, IL-8 results in the chemoattraction of neutrophils, the release of granule enzymes, and respiratory burst of neutrophils[54].

In nearly all cell types, LPS, TNFα, and IL-1β also upregulate the expression of inducible nitric oxide synthase (iNOS)[55]. iNOS exerts indirect anti-microbial and anti-viral effects in low concentrations and is an essential regulator of homeostasis[56,57]. However, overproduction of iNOS leads to cytotoxicity and mediates cell apoptosis, promotes tumor angiogenesis, and can facilitate disease states and tissue damage[58].

LPS and Post-weaning Syndrome

Following weaning, changes in diet and environment induce a fasting period in the piglets, which leads to decreased *Lactobacilli* in the gastrointestinal tract, thereby allowing increased populations of *E. coli* and shedding of LPS. Increased LPS acts through TLR4 to activate NF-κB and lead to the release of pro- and anti-inflammatory mediators. The release of pro-inflammatory cytokines causes morphological and functional changes in the gastrointestinal tract[59]. TNFα and IL-8 negatively influence the permeability of intestinal cells and the transport of ions and nutrients[60,61]. Specifically, TNFα gene expression decreases Na^+ / K^+ -ATPase activity leading to fluid accumulation in the small intestine and is a significant driver behind post-wean diarrhea[62,63]. Released LPS also causes structural changes in the small intestine by disrupting the structure of tight junctions and causing the internalization of tight junction protein occludin, which allows pathogens to cross into systemic circulation[61,64]. TNFα increases intestinal permeability by disrupting expression of claudins and occludins and by altering the lipid environment of phospholipid membranes to displace tight junction proteins[65,66]. Increased bacterial translocation drives the inflammatory response, and continuous production of pro-inflammatory cytokines leads to the suppression of important intestinal anti-inflammatory mediators like IL-10. Overproduction of pro-inflammatory and suppression of anti-inflammatory cytokines modulate the immune response and lead to inappropriate activation of antigen-presenting cells and intolerance to specific antigens, as well as limiting the proliferation of T cells[52].

Inflammatory mediators play a vital role in initiating a more robust immune response, activating other immune cells, and maintaining homeostasis. However, it is clear that overexpression of the immune response is deleterious. Therefore, many LPS therapies are

targeted at increasing immune surveillance and antigen presentation while preventing the over elicitation of these inflammatory mediators.

Alkaline Phosphatase

Alkaline phosphatases (AP) are homodimeric enzymes that catalyze the hydrolysis of monoesters of phosphoric acid and transphosphorylation reactions. AP are naturally occurring in the mammalian body and are divided into four types: tissue non-specific AP (TNAP), placental AP (PLAP), germ cell AP (GCAP) and intestinal AP (IAP)[67]. Intestinal alkaline phosphatase is a glycoprotein anchored in the apical membrane of the small intestine and is found in the highest levels in the duodenum, followed by the jejunum and ileum. IAP has several biological roles, including being a negative regulator of intestinal fat absorption, maintaining bicarbonate secretion and pH balance, and exerting immune-protective effects[68,69]. Expression of IAP is dependent upon enterocyte differentiation; therefore, the enzyme is often used as a biomarker for abnormal digestive and absorptive functions in the small intestine.

Specific proteins in tight junction adhesion complexes reside in lipid rafts on the apical membrane of the intestinal epithelial cells, and during lipid absorption, IAP is located in the same area. Lipid rafts are a unique subdomain of the plasma membrane and are enriched in glycosphingolipids, cholesterol, and sphingomyelin. When Caco-2 cells, an intestinal cell line, were treated with the pro-inflammatory cytokine IFN-γ, the disruption of lipid rafts preceded intestinal permeability failure[70]. The study also confirmed this in dextran sulfate sodium (DSS) induced colitis in mice and human ulcerative colitis patients. Treatment of another intestinal epithelial cell line, T84, with TNFα and IFN-γ resulted in altered lipid composition of lipid rafts and loss of barrier function by displacing occludins[65]. Another study determined that these lipid

rafts were released from the tips of the microvilli of intestinal epithelial cells and concluded that the shedding is one mechanism for distributing IAP activity throughout the mucosal layer of the gut[71]. Finally, the same study also reported that both IAP expression and luminal vesicle production and shedding upregulated in the presence of Gram-negative bacteria.

The toxicity of LPS, specifically the lipid A moiety, is dependent upon its phosphorylation state[72]. Since IAP is found in the small intestine and catalyzes the hydrolysis of phosphoric groups, and the toxicity of LPS is dependent upon the phosphorylation state, it stands to reason that IAP could dephosphorylate LPS.

The ability of IAP to dephosphorylate LPS, at physiological pH, and to reduce the toxic effects *in vivo* was first shown in 1997[73,74]. A significant role of alkaline phosphatase *in vivo* is to detoxify bacterial LPS that is present in the intestinal lumen. High levels of IAP can control LPS-induced inflammation in two ways: by dephosphorylating LPS and decreasing TLR4 stimulation, and by preventing NF-κB translocation to the nucleus by inhibiting the phosphorylation of two critical proteins in the pathway, IκBα and RelA/p65[75]. IAP activity has been shown to increase in the presence of Gram-negative bacteria or with LPS alone, and Gram-positive bacteria that lack LPS did not affect IAP gene expression[76]. The efficacy of IAP to dephosphorylate LPS and reduce its toxic effects have been shown many times in experiments with IAP administered intra-peritoneal and attenuating LPS toxicity[75,76]. Furthermore, oral administration of IAP prevented GIT tissue damage and pro-inflammatory cytokine expression that was induced by DSS in mice[77].

During weaning, piglets go through a fasting period as their immature digestive and immune systems are maturing, and it is well known that fasting dramatically decreases IAP activity[78]. Expression of IAP was also significantly reduced in weaned piglets compared to

suckling, and this contributes to the increased occurrence of GIT pathogens[79]. Fasting also decreases *Lactobacillus* populations which allow overpopulation of Gram-negative bacteria, and treatment with the probiotic *Lactobacillus casei* stimulated IAP activity[80]. IAP's ability to reduce bacterial translocation and attune commensal microbiota may be an ancillary effect of pH regulation, as an alkaline microenvironment is unfavorable for the growth of pathogens[68].

Porcine epidermal growth factor (pEGF) is found in sow milk and contributes to postnatal gut mucosal growth and development, and a recent study showed that supplementing weaned pigs with exogenous pEGF caused an increase in gene expression and protein activity of digestive enzymes in the GIT, including IAP[81,82]. Therefore, weaning contributes to a decrease in expression and activity of immuno-protective enzyme intestinal alkaline phosphatase, but exogenous supplementation exerts protective effects against LPS-mediated disease.

Monophosphoryl Lipid A

LPS contains two phosphate groups in the lipid A moiety, and interactions with alkaline phosphatases cause the release of inorganic phosphate and the formation of monophosphoryl lipid A (MPLA)[74,83]. The absence of the 1-phosphate on MPLA is believed to weaken the dimerization of TLR4/MD2, which presumably induces a structural change in the TLR4 receptor complex that alters the recruitment of adaptor proteins[84]. MPLA has distinct signaling properties as it predominantly activates the TLR4/TRAM-TRIF pathway over the more inflammatory MyD88-dependent pathway[85]. This difference in observed effects after exposure to LPS or MPLA could be explained as an active suppression, rather than a passive loss, of pro-inflammatory activity. MPLA is classified as a TLR4 agonist, meaning it signals through TLR4/MD2 and maintains the immuno-stimulatory properties of LPS but with reduced toxicity

and elicited inflammatory response. Specifically, MPLA increases antigen presentation of antigen presenting cells, like dendritic cells or macrophages, and subsequent activation of the MHC in effector T and B cells[86].

The gut epithelium has specialized epithelial cells called microfold cells (M cells) that transport luminal antigens and bacteria to underlying antigen presenting cells and reside in the follicle-associated epithelium surrounding Peyer's patches. Peyer's patches are aggregated lymphoid follicles and are part of the gut-associated lymphoid tissue (GALT), which consists of both aggregated and isolated lymphoid follicles[87]. The GALT accounts for about 70% of the body's immune system and is the largest lymphoid organ. Exposing M cells to MPLA increases their ability to uptake antigens and present them to lymphocytes in the GALT, which is vital to mounting humeral and cell mediates responses[88]. Furthermore, increasing the antigen uptake ability of one component of the GALT may increase the antigen presentation ability of the entire tissue. Due to its ability to increase antigen uptake and presentation of APC, MPLA dramatically enhances the efficacy of mucosal delivered vaccines and is currently being used as a vaccine adjuvant[89,90].

Summary

Weaning is the most stressful part of a pigs' life due to extreme and sudden environmental and physical stressors. These effects can lead to detrimental morphological and functional changes in the gastrointestinal tract, which promote microbial dysbiosis, increased gut permeability and bacterial translocation, malnutrition, and inflammation. The sudden increase in pathogenic bacteria, such as *E. coli* and *Salmonella*, leads to increased TLR4 stimulation and cytokine release; promote more inflammation, diarrhea and decreases immune function. LPS toxicity is dependent upon its phosphorylation state, and intestinal alkaline phosphatase has been shown previously to dephosphorylate LPS and change its ability to bind TLR4 resulting in altered signal transduction, thus reducing the innate immune response.

Current literature on the benefits of alkaline phosphatase and monophosphoryl lipid A utilizes the mammalian IAP isoform which is often derived from swine or bovine. Obtaining IAP from a mammalian host requires euthanizing the animal and scraping the mucosa of the small intestine, followed by various digestion and isolation techniques. This process requires land and money to house the animals and feed for them, produces negative environmental output, and has enzymatic variability between animals due to diet, disease state, and genetics. Furthermore, it requires large numbers of animals for a small amount of enzyme. Overall, it results in a high economic and environmental cost for a highly variable product.

Microbial enzymes are used in a variety of fields and have a large number of biotechnological applications. Bacterial hosts can be engineered to rapidly and efficiently overexpress recombinant enzymes and then cultured in large quantities to produce a substantial amount of the desired enzymes[91]. The fast growth rate and simple requirements of microbes make them a more sustainable, economically, and environmentally friendly option. Microbial-

derived enzymes are rapidly becoming more popular than conventional enzymatic production methods due to their consistency, ease of optimization, regular supply, and greater catalytic activity[92]. Recently, a microbe-derived alkaline phosphatase has been produced that purportedly can detoxify Gram-negative bacteria LPS and provide the same GIT benefits and immune-stimulatory effects as mammalian-derived IAP.

Therefore, it stands to reason that exogenous supplementation of a microbe-derived AP could interact with the increased populations of Gram-negative bacteria associated with weaning and detoxify their LPS products and strengthen gastrointestinal barrier function. This interaction would lead to a decreased inflammatory response, decreased morphological changes, microbiome symbiosis, increased nutrient absorption and ion channel usage, and an overall decreased mortality and increased growth rate during weaning. The goal of this thesis was to determine the ability of microbe-derived AP (MAP) to reduce the toxic effects of LPS *in vitro* and *in vivo*, and if exogenous supplementation of MAP during the post-weaning phase would result in increased intestinal IAP expression and activity, and protect against post-weaning syndrome-induced inflammation and GIT perturbations.

CHAPTER 2: MICROBIAL-DERIVED ALKALINE PHOSPHATASE DETOXIFIES LIPOPOLYSACCHARIDE AND ALTERS SICKNESS BEHAVIOR AND INFLAMMATORY RESPONSE IN WEANING PIGS

2.1 Abstract

Mammalian-derived intestinal alkaline phosphatase (IAP) detoxifies lipopolysaccharide (LPS) but it is not readily available for commercial application. Therefore, we sought to determine the ability of a microbial-derived alkaline phosphatase (MAP) to detoxify LPS and reduce the inflammatory response *in vitro* and *in vivo.* Compared to *Salmonella enterica* LPS or *Escherichia coli* LPS, MAP-detoxified LPS-stimulated primary alveolar macrophages had reduced transcription of pro-inflammatory cytokines *TNFα, IL-6, and IL-1β*, and anti-inflammatory cytokine *IL-10* ($P<0.001$). Weaned piglets injected i.p. with MPLA or saline had no differences in serum TNFα, IL-6, IL-10, or IL-4 concentrations. LPS-injected piglets had higher serum TNFα ($P=0.005$) and IL-6 ($P=0.006$) levels than control piglets at peak-challenge, and LPS-induced TNFα remained elevated 4 hours post-injection ($P=0.003$). LPS injected piglets had serum IL-10 and IL-4 as compared to control ($P=0.025$; $P=0.045$) and MPLA treated pigs ($P=0.006$; $P=0.05$). LPS treatment upregulated TNFα transcript in the spleen, as compared to control ($P=0.006$) and ($P=0.029$) MPLA treated piglets. LPS treatment caused sickness behavior as measured by decreased feed intake ($P=0.031$) and lethargy; however, MPLA did not induce behavioral changes. Furthermore, novel findings comparing IAP and MAP, show that MAP has an increased efficacy of detoxifying LPS and ameliorating macrophage inflammatory responses *in vitro.* IAP-detoxified LPS-treated primary alveolar macrophages had increased gene expression of *TNFα, IL-1β, and IL-10* compared to cells stimulated with MAP-detoxified LPS ($P<0.001$). Together these results support the efficacy of MAP to detoxify both *S. enterica* and *E.*

coli LPS and reduce the toxic effects of LPS in primary cultured alveolar macrophages and in weaning piglets.

2.2 Introduction

Bacterial endotoxins are associated with sepsis and septic shock and potentiate other diseases such as Crohn's disease, cystic fibrosis, and heart disease[93]. Lipopolysaccharide (LPS) is one such endotoxin and is present on the cell wall of Gram-negative bacteria, such as *Salmonella enterica* and *Escherichia coli*. Upon gastrointestinal infection, LPS can be spontaneously released into host circulation and initiate an inflammatory response marked by fever, decreased feed intake, and increased diarrhea[35,36]. In neonates the immature immune system can be overcome, resulting in an overproduction of pro-inflammatory cytokines that modulate the immune response and disrupt tight junction proteins in the gut, leading to further microbial translocation[64–66]. The innate immune response is initiated when homeostatic functions are perturbed and surveilling immune cells recognize patterns associated with these perturbations, called danger-associated molecular patterns (DAMP) and pathogen-associated molecular patterns (PAMP). PAMP and DAMP bind to specific receptors that influence gene expression and regulate the immune functions of clearance, autophagy, phagocytosis, and, through downstream signaling molecules, recruit other immune cells.

Toll-like receptors (TLR) are transmembrane proteins found on cells that contribute to inflammatory responses, such as macrophages or epithelial cells of mucous membranes, which recognize PAMP and DAMP and mediate inflammatory effects through downstream signaling pathways. TLR4 recognizes LPS and initiates the innate immune systems' inflammatory response[35,36]. LPS is structurally organized into an outer polysaccharide region containing the O antigen; and an inner lipid moiety containing lipid A. Specifically, TLR recognize the lipid A region of LPS[37]. TLR4 signaling, through MyD88 or TRIF/TRAM signaling pathways, ultimately results in a rapid burst of pro-inflammatory cytokines into the surrounding tissue and

circulation and recruits effector cells, such as neutrophils, to travel to the site of infection for assistance in the clearance of the pathogen. The major pro-inflammatory cytokines that are secreted following an LPS challenge are TNFα, IL-1β, and IL-6. IL-1β and TNFα share several biological roles, such as activating neutrophils, and high concentrations in the circulation of these two cytokines can result in numerous pathophysiological effects such as fever and multiple organ failure[46–48]. IL-6, while still pro-inflammatory, is considered to be less potent than TNFα or IL-1β, and can be induced by those two cytokines. IL-6 has also been implicated in multiple organ failure. IL-4 and IL-10 are two major anti-inflammatory cytokines released following TLR4 stimulation. IL-4, mainly synthesized by activated T-lymphocytes, has biological roles in B cell proliferation and inhibition of inflammatory cytokine production[50,51]. IL-10 diminishes excessive immune reactions, reduces antigen presentation, and inhibits T cell activation [52]. However, overproduction of IL-4 and IL-10 can slow pathogen clearance and potentiate immunopathology.

Mammalian enteric brush border enzyme intestinal alkaline phosphatase (IAP) has been shown to exert protective effects in the gut, by detoxifying LPS in the intestinal lumen. IAP is a glycoprotein anchored in the apical membrane of the small intestine and has several biological roles including being a negative regulator of intestinal fat absorption, maintaining bicarbonate secretion and pH balance, and exerting immune-protective effects[68,69]. The toxicity of LPS is highly dependent upon its phosphorylation state, and removal of one of the two phosphate groups by IAP reduces the toxic effects of LPS *in vitro* and *in vivo*[73,74]. High levels of IAP can control LPS-induced inflammation in two ways: by dephosphorylating LPS and decreasing TLR4 stimulation, and by preventing NF-κB translocation to the nucleus by inhibiting the phosphorylation of two critical proteins in the pathway, IκBα and RelA/p65[75]. When an alkaline

phosphatase removes the 1-phosphate on LPS, it yields monophosphoryl lipid A (MPLA)[83,84]. MPLA is known to activate the TLR4/TRAM-TRIF pathway predominantly over the more inflammatory MyD88-dependent pathway[85]. MPLA works as a TLR4 agonist and maintains the immune-stimulatory properties of LPS, but with reduced toxicity and inflammatory response. MPLA has been shown to increase antigen presentation of circulating antigen presenting cells and M cells in gut-associated lymphoid tissue[86,88].

Microbial enzymes are utilized in a variety of fields and have a large number of biotechnological applications. Bacterial hosts engineered to rapidly and efficiently overexpress recombinant enzymes can be used to produce a substantial amount of the desired enzyme[91]. Microbial-derived enzymes are quickly becoming more popular than conventional enzymatic production methods due to their consistency, ease of optimization, regular supply, and greater catalytic activity[92]. Recently, a microbe has been manipulated to produce alkaline phosphatase that can purportedly detoxify Gram-negative bacteria LPS and provide the same GIT benefit and immune-stimulatory effects as IAP. The objectives of the study were to assess the ability of a microbial-derived alkaline phosphatase (MAP) to detoxify LPS at physiological pH and reduce the inflammatory effects of LPS, as well as the potential immuno-stimulatory properties of MPLA. Using mammalian-derived IAP, we also tested whether MAP would have greater detoxification ability than IAP, and if the subsequent MPLA products would have differences in their elicited inflammatory responses. We hypothesized that MAP would detoxify LPS and elicit lower levels of pro-inflammatory cytokines from both the immortalized RAW264 cell line and primary pig alveolar macrophages. We also hypothesized that i.p. administration of MPLA would result in lower levels of circulating cytokines and decreased sickness behavior in weaning piglets, compared to LPS.

2.3 Materials and Methods

Isolation of primary alveolar macrophages

Pulmonary organs with the trachea clamped were obtained from 6-month old cross-bred Yorkshire-Landrace male barrows from the University of Illinois Meats Laboratory. The lungs were filled with 500 mL of RPMI 1640 supplemented with 500 IU/mL penicillin/streptomycin. Lungs were massaged approximately 2 minutes, and the lavage fluid was aspirated with a 25-mL pipette and collected in a sterile 50 mL conical tube. The lavage was repeated twice and always yielded a white, foamy fluid. The collected suspension was centrifuged at 4°C at 400×g for 10 minutes and the supernatant discarded. The cell pellet was washed with 2.0 mL of ammonium chloride (0.83% NH_4Cl in 0.1% $KHCO_3$-0.01 M EDTA) to lyse red blood cells. The suspension was then washed three times with RPMI-1640 plus 100 IU/mL penicillin/streptomycin. Following washes, the pellet was suspended in RPMI 1640 containing 10% FBS, 4mM HEPES, 1× minimum essential medium (MEM), nonessential amino acids, 1 mM sodium pyruvate, and 100 IU/mL penicillin/streptomycin. Cells were seeded onto 12-well culture plates at a concentration of 10^6 / well and allowed to adhere for 24 hours in a humidified incubator at 5% CO_2 and 40°C, then washed twice to remove cellular debris and non-adherent cells. The cells were then treated as alveolar macrophages and allowed to rest for 2 hours prior to stimulation.

RAW264 and primary alveolar macrophage culture and treatments

Initially, the immortalized pig alveolar macrophage cell lines CRL-2843/CRL-2844/CRL-2855 (ATCC) were utilized but failed to develop normal morphology during standard growth periods and had an aberrant inflammatory response when treated with LPS **(Supplemental Fig 2.1).** Therefore, it was determined to use immortalized murine macrophage

cell line RAW264 (ATCC® TIB-71) for all initial experiments. Preliminary studies with RAW264 showed that 100 ng/mL *S. enterica* LPS or *E. coli* LPS was the optimal concentration for eliciting a robust inflammatory response without killing the cells. The viability of RAW264 and porcine alveolar macrophages was evaluated using the Thermo Scientific Pierce Lactate Dehydrogenase Cytotoxicity Assay Kit (Thermo Scientific, Rockford, IL, USA) in accordance with the manufacturer's instructions was and was <10%. A similar dose-response curve was created for treating RAW264 with MAP to determine if the new compound was toxic. Finally, LPS was incubated with increasing concentrations of MAP. Incubating LPS and MAP in a ratio of 1 IU/mL MAP: 1 µg/mL LPS was determined to create optimum detoxification. Both *S. enterica* and *E. coli* LPS serotypes were used and are presented in separate figures for clarification.

RAW264 cells were maintained in 75-cm^2 tissue culture flasks (TPP) in DMEM supplemented with 10% FBS and 100 units/mL penicillin/streptomycin at 37°C in a humidified incubator under 5% CO2. For stimulation, cells were plated in 12-well culture plates overnight at a concentration of 10^6 / well. Cells were then washed and allowed to rest for two hours prior to treatment. RAW264 cells were stimulated with 100 ng/mL *Salmonella enterica* serotype Typhimurium LPS (L2262; Sigma-Aldrich, St. Louis, MO, USA), 0.10 IU/mL MAP (Elanco Animal Health, Greenfield, IN, USA), 0.10 IU/mL IAP (P0114; CAS 9001-78-9; EC 3.1.3.1; Sigma-Aldrich), 100 ng/mL IAP-derived MPLA (iMPLA), or 100 ng/mL MAP-derived MPLA (MPLA; see LPS Challenge). In separate but identical trials, RAW264 cells were stimulated with 100 ng/mL of *E. coli* LPS (L3137; serotype 0127:B8, Sigma-Aldrich) or detoxified *E. coli* LPS (MPLA). IAP-derived MPLA was prepared using the same *S. enterica* or *E. coli* serotypes and protocol as listed under Piglet Immune Challenge. Primary pig alveolar macrophages were plated

on 12-well plates at a concentration of 10^6 / well and supplemented with 10% FBS, 100 IU/mL penicillin/streptomycin and maintained at 40°C in a humidified incubator under 5% CO2. Primary alveolar macrophages were stimulated for four hours with 100 ng/mL *S. enterica* LPS, 0.10 IU/mL MAP, 0.10 IU/mL IAP (Sigma), 100 ng/mL IAP-derived MPLA, or 100 ng/mL MAP-derived MPLA. In separate but identical trials, primary alveolar macrophages were stimulated with 100 ng/mL *E. coli* or detoxified versions of *E. coli* LPS. Cells were harvested 4 hours post-treatment, and both media and cells were collected, and cells were put directly in Trizol reagent (Invitrogen, Grand Island, NY, USA).

Animals

Eighteen Yorkshire piglets from the University of Illinois swineherd were naturally farrowed and weaned at 21 days of age. Results are from three separate cohorts. After weaning, piglets were kept in a 312 cm L × 130 cm W × 80 cm H wooden pen in sex-matched littermate pairs. Piglets were kept on the premises of University of Illinois Swine Research Center and allowed 48 hours to acclimate before injections. Room temperature was kept at 28°C and piglets were provided supplemental heat. After completion of the study (postnatal day 23, 4 hours post-injection) piglets were anesthetized using a telazol:ketamine:xylazine solution (50 mg of tiletamine plus 50 mg of zolazepam reconstituted with 2.5 mL ketamine [100 mg/mL] and 2.5 mL xylazine [100 mg/mL]; Fort Dodge Animal Health, Fort Dodge, IA); the anesthetic combination was administered i.m. at 0.03 ml/kg BW. After verifying anesthetic induction, piglets were euthanized via i.c. administration of sodium pentobarbital (1 mL/4.5 kg BW; Fatal Plus®, Vortech Pharmaceuticals, Dearborn, MI, USA). All animal procedures were approved by the University of Illinois at Urbana-Champaign Institutional Animal Care and Use Committee.

LPS Challenge

Piglets were divided into three treatment groups for injections controlling for sex, litter of origin, and body weight: vehicle, lipopolysaccharide (LPS), and monophosphoryl lipid A (MPLA). *Salmonella enterica* serotype Typhimurium LPS (L2262; Sigma-Aldrich) was utilized for the LPS treatments of piglets. To dephosphorylate LPS into MPLA, LPS was incubated with MAP, supplemented with 1.0 mM $MgCl^{2+}$ and 0.1 mM zinc sulfate monohydrate, at pH 8.5 in the ratio of 1 IU MAP:1 µg LPS, as previously described by Bates et al.[94]. In summary, LPS was incubated with MAP for 4 hours at 40°C. Filter-sterilized solutions of LPS and vehicle were mock-treated by being incubated under the same conditions without the addition of MAP. After the final incubation, the LPS-MAP mixture was treated as monophosphoryl lipid A (MPLA). LPS and MPLA were injected i.p. at a dose of 10 µg LPS/kg BW. Previous work in our lab has shown this dose of LPS induces an increase in plasma TNFα that peaks at 4 hours post-injection[95]. Vehicle was administered i.p. at the same volume/BW. Treatments were administered at 0800 for all cohorts. Piglets were euthanized 4 hours post-injection and serum collected at 0, 2, and 4 hours post injection to measure circulating pro- and anti-inflammatory cytokines

Behavior

Behavior was digitally recorded throughout the four-hour time period using a Sony DCR SR300 camera mounted above the pen. Time spent eating and inactive were estimated from the video records by instantaneous scan sampling at one-minute intervals. Behaviors were scored by a trained experimenter blinded to treatment. A pig was considered to be eating if standing with head in the trough, and a pig was considered inactive if it was lying down. Feed was removed 3

hours prior to injections and placed back in pen 45 minutes post-injection to control for variation in food motivation.

Gene Expression

Total RNA from macrophages were isolated using the Tri Reagent protocol (Invitrogen). Complimentary DNA was synthesized using a high-capacity RT kit (Applied Biosystems, Grand Island, NY, USA) according to the manufacturer's instructions. Quantitative real-time PCR was performed using the Applied Biosystems Taqman Gene Expression Assay protocol, and relative gene expression was determined from the fluorescent data on an ABI PRISM 7900HT sequence detection system (PerkinElmer, Waltham, MA, USA). All mouse primers for quantitative PCR were obtained from Integrated DNA Technologies (Coralville, IA, USA). Genes of interest **(Supplemental Table 2.1)** were compared against reference controls. RAW264 cells were analyzed with the reference gene GAPDH (NM_008084), and primary alveolar macrophages were compared to reference gene RPL19 (Ss03375624_g1). PCR conditions included 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds, and 1 cycle at 60°C for 1 minute. Gene expression was determined using the $2^{-\Delta\Delta Ct}$ calculation method as previously described[96]. Data are expressed as fold change versus controls. Tissue collected from the piglets at the end of the 4-hour time-point were also used to assess steady-state mRNA levels utilizing the method and primers mentioned above for primary pig alveolar macrophages. In brief, spleen and liver samples were collected and flash frozen. Tri Reagent protocol (Invitrogen) was used for total RNA isolation with 50 mg/tissue per animal. Complementary DNA was synthesized, and RT-qPCR ran for relative gene expression of TNFα, IL-1β, IL-6, and IL-10. RPL-19 was

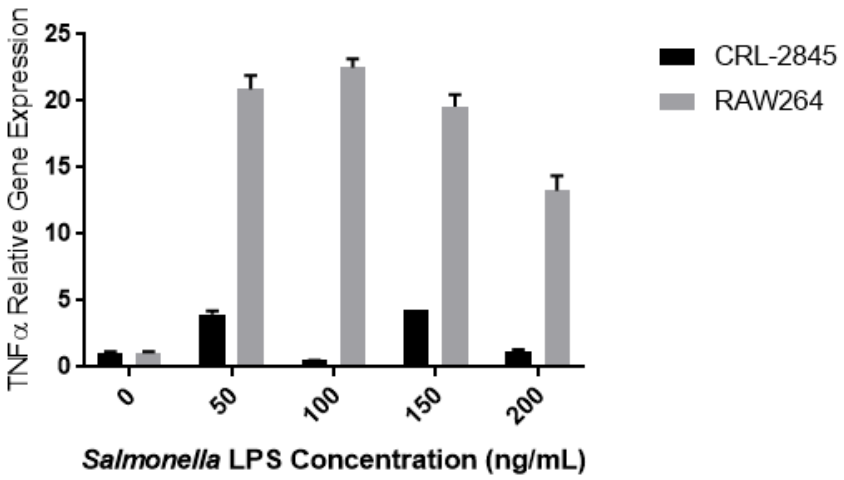
used as a housekeeping control gene and data compared using the $2^{-\Delta\Delta Ct}$ calculation method[96], with data presented as fold change versus control.

Cytokine Measurements

ELISA were used to determine serum and culture medium concentrations of IL-10, IL-4, IL-6, and TNFα according to manufacturer's instructions (R&D Systems; Minneapolis, MN, USA and Thermo Fisher; Carlsbad, CA, USA). The interassay coefficient of variance was <5% for all assays conducted.

Statistics

Data were analyzed utilizing one-, two-, or three-way ANOVA with or without repeated measures, followed by Tukey's multiple comparisons tests when appropriate. Statistical significance was set at $p \leq 0.05$. Analysis was performed using GraphPad Prism version 7.00 for Windows (GraphPad Software, La Jolla, CA) or SAS™ 9.4 software (SAS Institute, Cary, NC, USA). All data are expressed as means ± standard error of the mean. Treatments with different letters are significantly different.



Supplemental Fig 2.1. Representative graph showing differences in TNFα gene expression from *Salmonella* LPS treated CRL-2845 and RAW264 cells. CRL cell lines failed to develop normal morphoology during standard growth peroids and had an aberrant inflammatory response when treated with LPS. (n = 8)

Supplemental Table 2.1. Quantitative real-time PCR primer information

Gene	Classification	Species	Accession number[a]	Assay identification[b,c]
GAPDH	Reference	mouse	NM_008084	Mm.PT.39a.1[b]
TNFα	Pro-inflammatory	mouse	NM_013693	Mm.PT.58.29509614[b]
IL-6	Pro-inflammatory	mouse	NM_031168	Mm.PT.58.13354106[b]
IL-1β	Pro-inflammatory	mouse	NM_008361	Mm.PT.58.41616450[b]
IL-10	Anti-inflammatory	mouse	NM_010548	Mm.PT.58.23604055[b]
RPL-19	Reference	pig	AF435591	Ss03375624_g1[c]
TNFα	Pro-inflammatory	pig	NM_214022	Ss03391318_g1[c]
IL-6	Pro-inflammatory	pig	NM_214399	Ss03384604_u1[c]
IL-1β	Pro-inflammatory	pig	NM_214055	Ss03393804_m1[c]
IL-10	Anti-inflammatory	pig	NM_214041	Ss03382372_u1[c]

[a]NCBI GenBank accession number.

[b]IDT Gene Expression Assay identification number.

[c]Applied Biosystems TaqMan Gene Expression Assay identification number.

2.4 Results

Microbial-derived alkaline phosphatase detoxifies *S. enterica* and *E. coli* LPS

It was previously reported that mammalian intestinal alkaline phosphatase (IAP) is capable of detoxifying LPS[75,77,94]. However, the ability of microbe-derived alkaline phosphatase to detoxify LPS is unknown. The method of detoxifying LPS, through interaction with alkaline phosphatase, was adapted from Bates *et al*[76] to determine the capacity of microbe-derived alkaline phosphatase (MAP) to detoxify LPS and reduce LPS-induced macrophage responses *in vitro.* For clarification, following the incubation steps described above, the MAP-LPS mixture was considered to be monophosphoryl lipid A or MPLA.

As previously reported by others, pre-treating *Salmonella* LPS with MAP resulted in downregulated mRNA in RAW264 macrophages for pro-inflammatory cytokines TNFα, IL-6, and IL-1β and anti-inflammatory cytokine IL-10 as compared to *Salmonella* LPS treatment alone (**Fig 2.1a-d)**. Furthermore, treatment of RAW264 macrophages with MAP alone had no effect as compared to vehicle control (P=0.999). Every cytokine mRNA assessed was higher in RAW264 cells treated with *Salmonella* LPS compared to control (P<0.005) while MPLA was not different from control. RAW264 supernatant was collected, and TNFα and IL-6 protein concentrations determined (**Fig 2.1e-f).** TNFα protein concentration was elevated by *Salmonella* LPS treatment (P<0.001) and to a lesser extent with MPLA treatment (P=0.019). *Salmonella* LPS treatment elevated TNFα protein levels as compared to MPLA (P=0.001). IL-6 protein concentration from *Salmonella* LPS treated RAW264 cells was elevated compared to controls (P<0.001) and MPLA treatments (P<0.001). *Salmonella* MPLA treatment was not inflammatory enough to cause a significant change from controls and had a lower concentration of IL-6 (P=0.240). Similar results were obtained using LPS derived from *E. coli*. RAW264 macrophages had higher cytokine

production from treatment with *E.coli* LPS as compared to control treatments for TNFα, IL-6, IL-1β, and IL-10 ($P<0.001$) (**Fig 2.2a-d)**. Furthermore, MPLA derived from *E.coli* LPS elicited lower mRNA expression than *E. coli* LPS treatment alone for TNFα ($P=0.003$), IL-6 ($P=0.04$), IL-1β ($P=0.037$), and IL-10 ($P=0.005$). The supernatant was collected, and ELISA performed for TNFα and IL-6 (**Fig 2.2e-f)**. *E. coli* LPS upregulated TNFα protein ($P<0.001$); however, detoxified *E. coli* or MPLA, elicited significantly less protein than LPS ($P=0.002$). *E. coli* LPS stimulated the release of IL-6 compared to MPLA treatment ($P=0.002$). These results indicate that MAP is adept at detoxifying LPS products from Gram-negative *Salmonella* and *E.coli* bacteria.

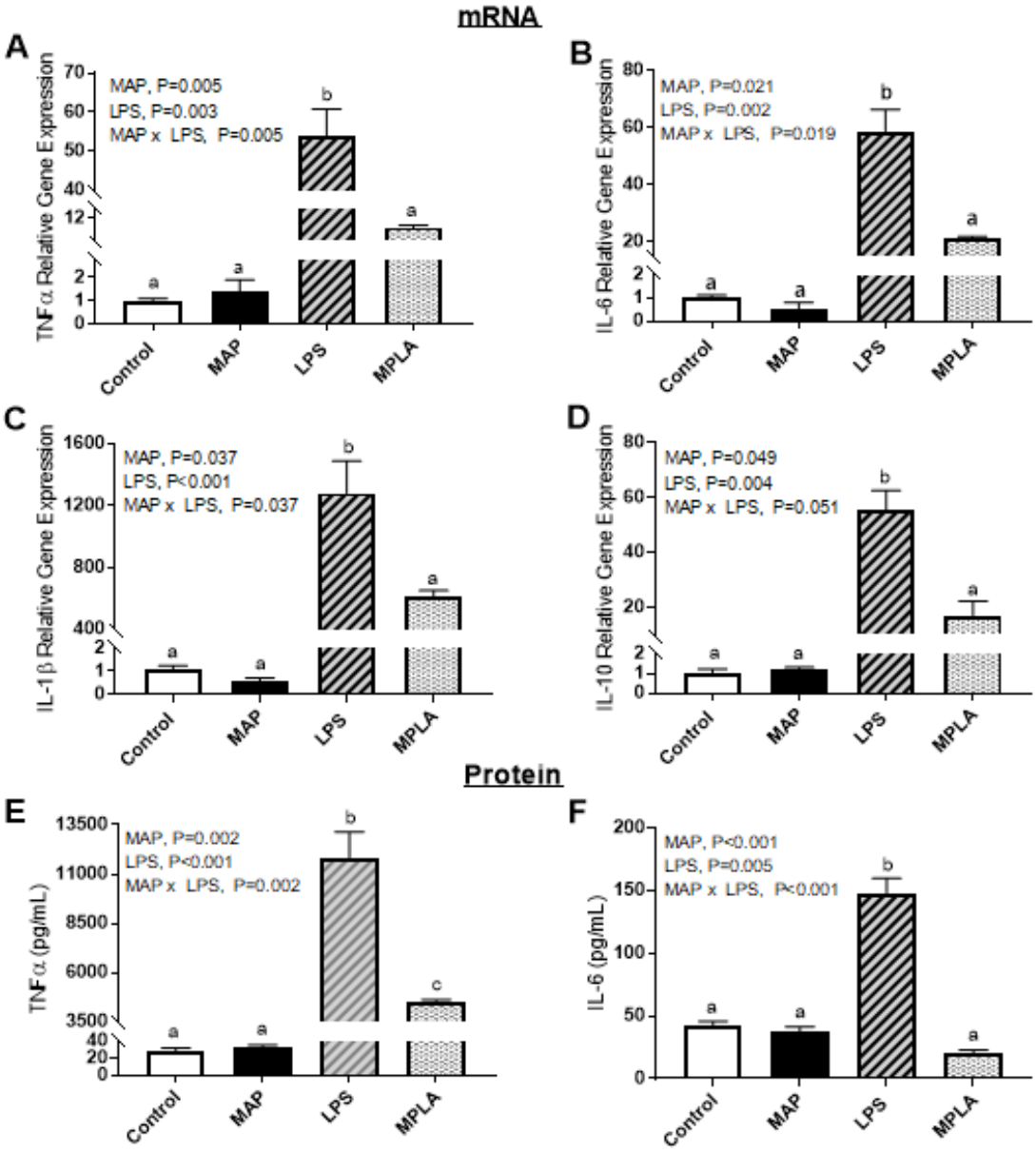


Fig 2.1. Gene expression of pro-inflammatory cytokines is increased in *Salmonella* LPS treated RAW264 cells, but not with MPLA treatment. RAW264 cells were treated with MAP, MPLA, or *Salmonella* LPS for 4 hours. (A) TNFα relative gene expression, (B) IL-6 relative gene expression, (C) IL-β relative gene expression, (D) IL-10 relative gene expression. (E) TNFα protein expression. (F) IL-6 protein expression. Treatments with different letters are significantly different. Bars represent the mean ± standard error of the mean (n = 2-6).

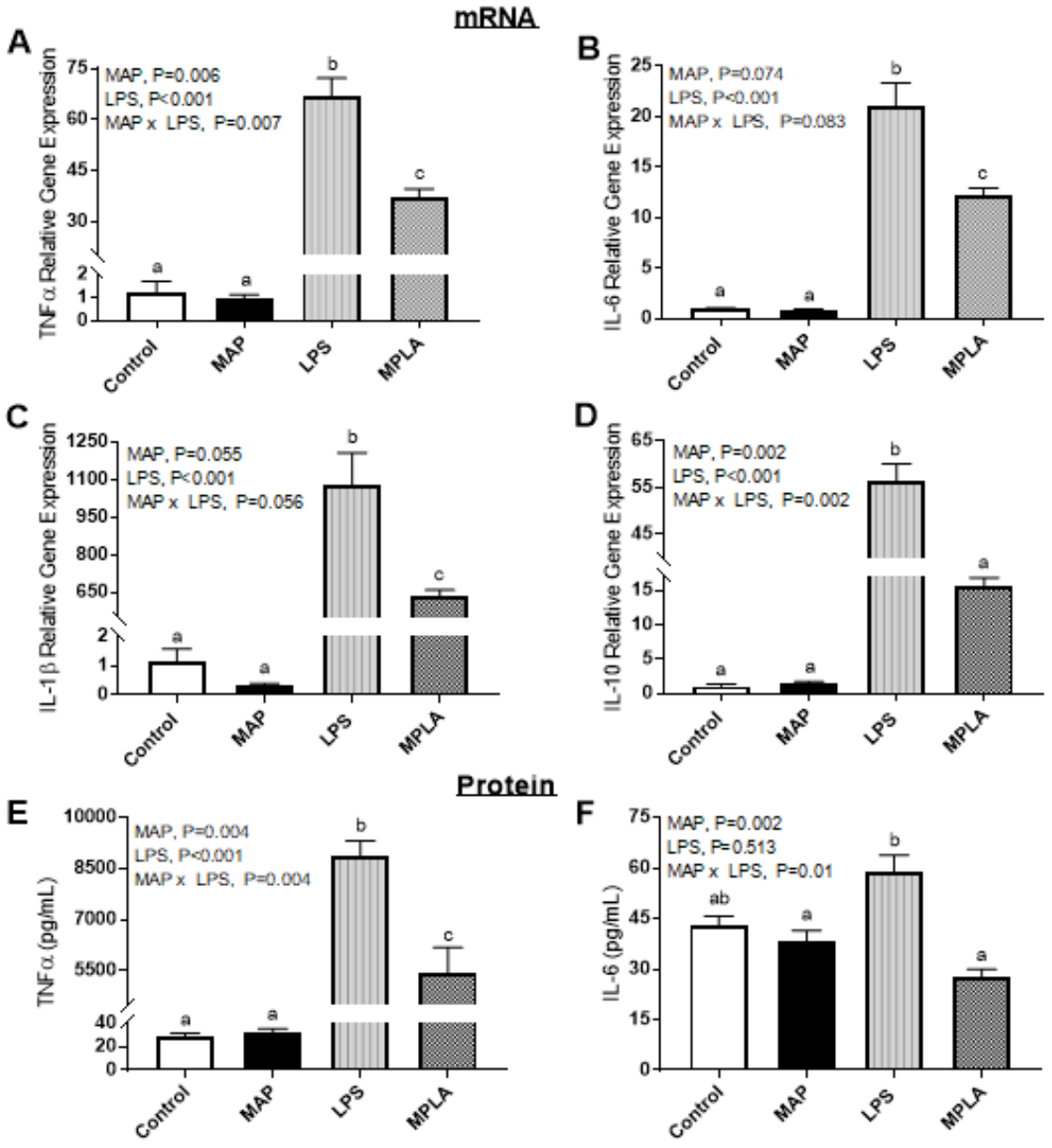


Fig 2.2. Gene expression of pro-inflammatory cytokines is increased in *E. coli* LPS treated RAW264 cells, but not with MPLA treatment. RAW264 cells were treated with MAP, MPLA, or *E. coli* LPS for 4 hours. (A) TNFα relative gene expression, (B) IL-6 relative gene expression, (C) IL-β relative gene expression, (D) IL-10 relative gene expression. (E) TNFα protein expression. (F) IL-6 protein expression. Treatments with different letters are significantly different. Bars represent the mean ± standard error of the mean (n = 2-6).

To confirm the results across species and in primary cells, primary pig alveolar macrophages were collected and treated with vehicle, 0.10 IU/mL MAP, 100 ng/mL *S. enterica* LPS, or 100 ng/mL of *Salmonella* MPLA. Cells and supernatant were collected to assess markers of inflammation (**Fig 2.3a-d)**. TNFα, IL-6, IL-1β, and IL-10 mRNA expression in primary alveolar porcine macrophages was upregulated with LPS ($P<0.001$) and MPLA treatment, however, LPS elicited a greater increase in cytokine expression compared to MPLA ($P<0.001$). These results demonstrate that MAP detoxifies *Salmonella* LPS, and the resultant MPLA product is less inflammatory. Porcine alveolar macrophage supernatant was subjected to ELISA to determine the protein concentration of IL-6 (**Fig 2.3e).** Both *Salmonella* LPS and *Salmonella* MPLA elevated IL-6 concentrations ($P<0.001$); however, *Salmonella* LPS treatment elicited more product than *Salmonella* MPLA treatment ($P<0.001$). Again, the above results were confirmed using *E. coli* LPS and MPLA derived from *E. coli* LPS. Compared to control treatment, *E. coli* LPS upregulated TNFα, IL-6, IL-1β, and IL-10 mRNA abundance in primary alveolar macrophages (**Fig 2.4a-d)**. Furthermore, for all measured cytokine gene expression, *E. coli* MPLA treatment was less inflammatory than *E. coli* LPS ($P=<0.001$). ELISA analysis of IL-6 protein in cell culture supernatant confirmed *E. coli* LPS treatment upregulated IL-6 protein as compared to control ($P<0.001$) and *E. coli* MPLA treatments ($P=0.001$) **(Fig 2.4e)**. Together these data suggest that MAP detoxifies *E. coli* LPS at physiological pH and temperature and that treatment with the product, MPLA, result in lower mRNA and protein levels of pro- and anti-inflammatory cytokines as compared to LPS.

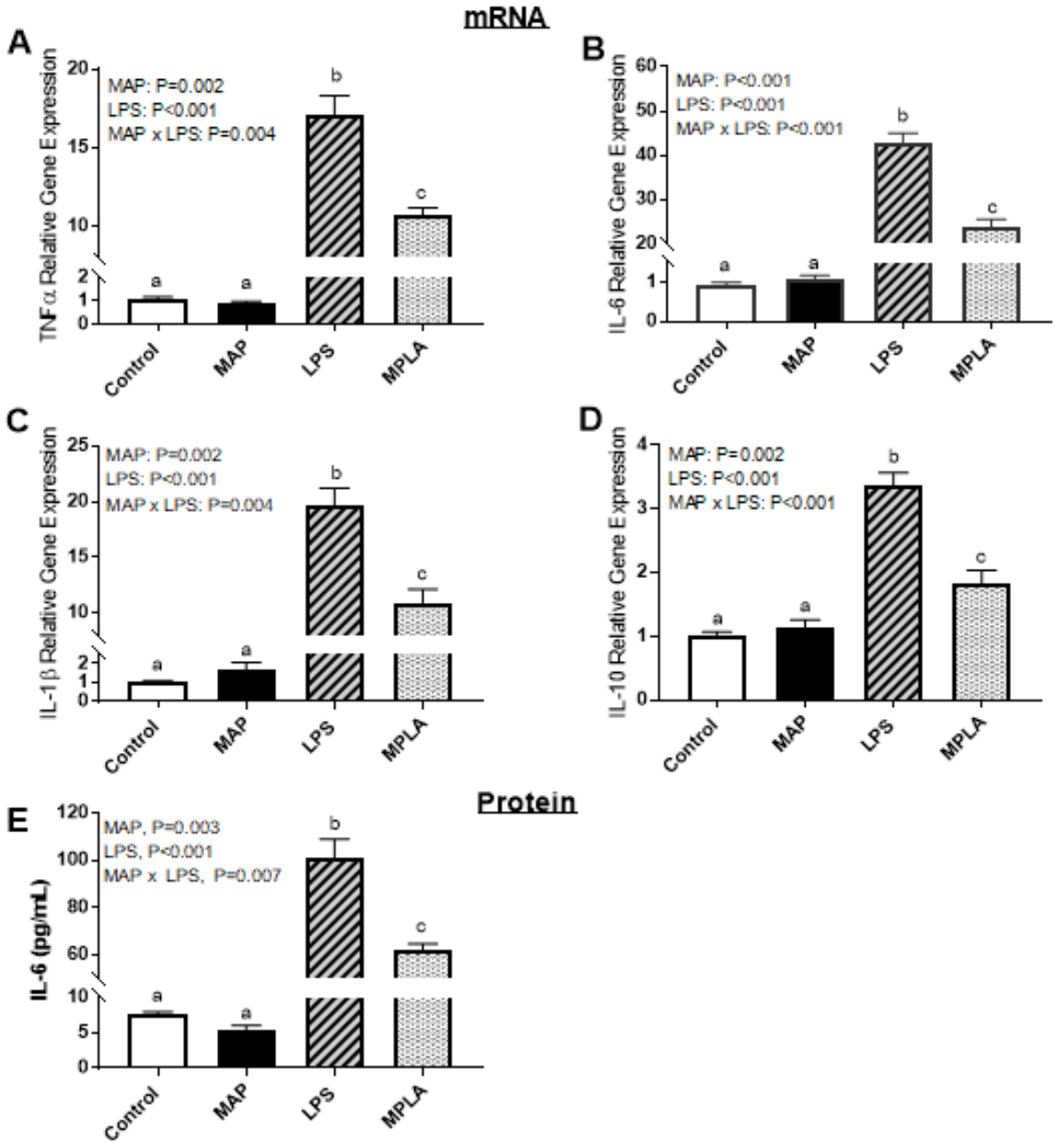


Figure 2.3. Increased pro-inflammatory cytokine expression in *Salmonella* LPS but not MPLA-treated primary cultured swine alveolar macrophages. MPLA-treated macrophages were significantly less stimulated than *Salmonella* LPS-treated as measured by mRNA abundance of TNFα (A), IL-6 (B), IL-1β (C), and IL-10 (D) and protein expression of IL-6 (E). Cells were treated with vehicle, MAP, *Salmonella* LPS, or MPLA for 4 hours. Bars represent the mean ± standard error of the mean (n = 7-11 for gene expression) (n = 3-4 for ELISA). Treatment means with different letters are significantly different.

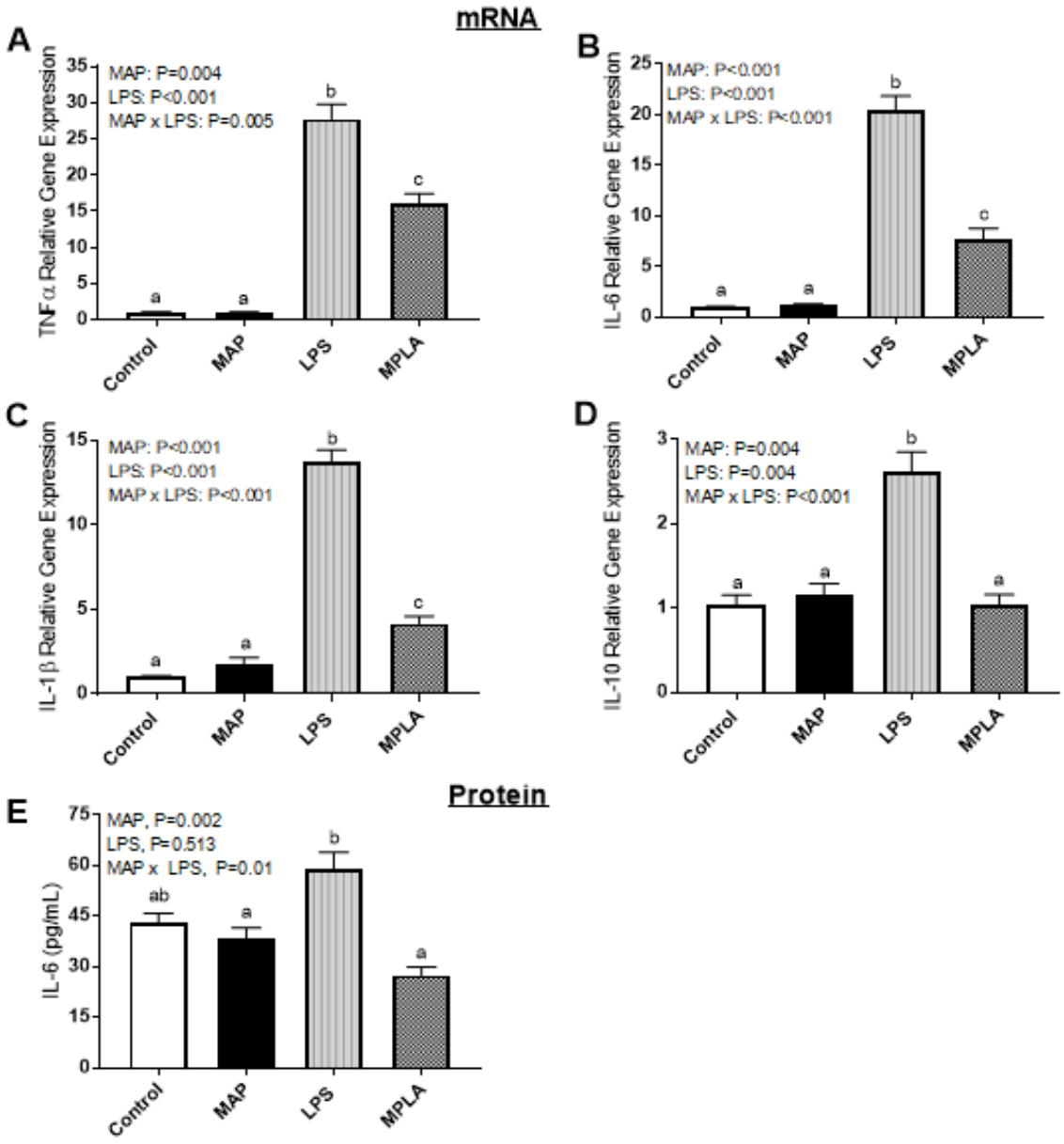


Figure 2.4. Increased pro-inflammatory cytokine expression in *E. coli* LPS but not MPLA-treated primary cultured swine alveolar macrophages. MPLA-treated macrophages were significantly less stimulated than *E.coli* LPS-treated as measured by mRNA abundance of TNFα (A), IL-6 (B), IL-1β (C), and IL-10 (D), and protein expression of IL-6 (E). Cells were treated with vehicle, MAP, *E. coli* LPS, or MPLA for 4 hours. Bars represent the mean ± standard error of the mean (n = 7-11 for gene expression) (n = 3-4 for ELISA). Treatment means with different letters are significantly different.

MAP differentially detoxifies both *Salmonella* and *E. coli* LPS as compared to mammalian-derived intestinal alkaline phosphatase.

Current literature supports the efficacy of mammalian-derived intestinal alkaline phosphatase (IAP) to detoxify LPS, and the resultant product, mono-phosphoryl lipid A (iMPLA) is recognized as a TLR4 agonist and vaccine adjuvant. However, there are no known results regarding the use of microbe-derived alkaline phosphatase or its subsequent product after incubation with LPS. To determine the ability of a microbe-derived alkaline phosphatase (MAP) to detoxify both *Salmonella* and *E.coli* LPS, compared to IAP, RAW264 and porcine alveolar macrophages were treated with IAP and iMPLA under the same conditions. Unexpectedly, MAP exceeded the ability of IAP to detoxify *Salmonella* LPS as iMPLA elicited a stronger pro-inflammatory response compared to MPLA (**Fig 2.5a-d)**. For RAW264 macrophages, there were no differences between control, MAP, or IAP treatments for all measured cytokines in the absence of either LPS or MPLA. TNFα mRNA levels from *Salmonella* MPLA treatment were not different from control (P=0.76). However, both *Salmonella* LPS and *Salmonella* iMPLA treatment resulted in higher TNFα gene expression (P<0.001). Surprisingly, there was no difference between LPS and iMPLA groups (P=0.99), but there was a difference between MPLA and iMPLA (P=0.001). These differences in elicited TNFα expression could be due to differential detoxifying properties of MAP and IAP. Comparable results were quantified for IL-6 gene expression. *Salmonella* LPS treatment elevated IL-6 (P<0.001) and was different from MPLA (P=0.001) but not iMPLA (P=0.299). IL-6 gene expression was not different between MPLA and iMPLA (P=0.092). *Salmonella* LPS and iMPLA caused a substantial increase in IL-1β expression (P<0.001), while *Salmonella* MPLA only moderately increased mRNA of IL-1β (P=0.027). Again, *Salmonella* MPLA elicited IL-1β expression was lower than *Salmonella*

iMPLA (P=0.027). IL-10 mRNA was increased with *Salmonella* LPS (P=0.003) and iMPLA (P=0.012), but not with MPLA treatment (P=0.605). *Salmonella* LPS treatment resulted in higher IL-10 expression than MPLA (P=0.006) but was not different from iMPLA (P=0.135). Furthermore, there was no difference in IL-10 mRNA between iMPLA and MPLA (P=0.254). ELISA confirmed TNFα and IL-6 results. *Salmonella* LPS, iMPLA, and MPLA treatment resulted in higher TNFα protein production (P<0.001; P<0.001; P=0.009) (**Fig 2.5e)**. Surprisingly, there were no differences between iMPLA and MPLA (P=0.413); however, *Salmonella* LPS treatment increased TNFα concentrations compared to MPLA and iMPLA (P<0.001). Only *Salmonella* LPS treatment caused an increase in IL-6 protein (P=0.007), although iMPLA treatment also raised IL-6 levels (**Fig 2.5f)**. *Salmonella* MPLA-induced IL-6 protein release was lower than LPS (P<0.001) and iMPLA (P=0.03).

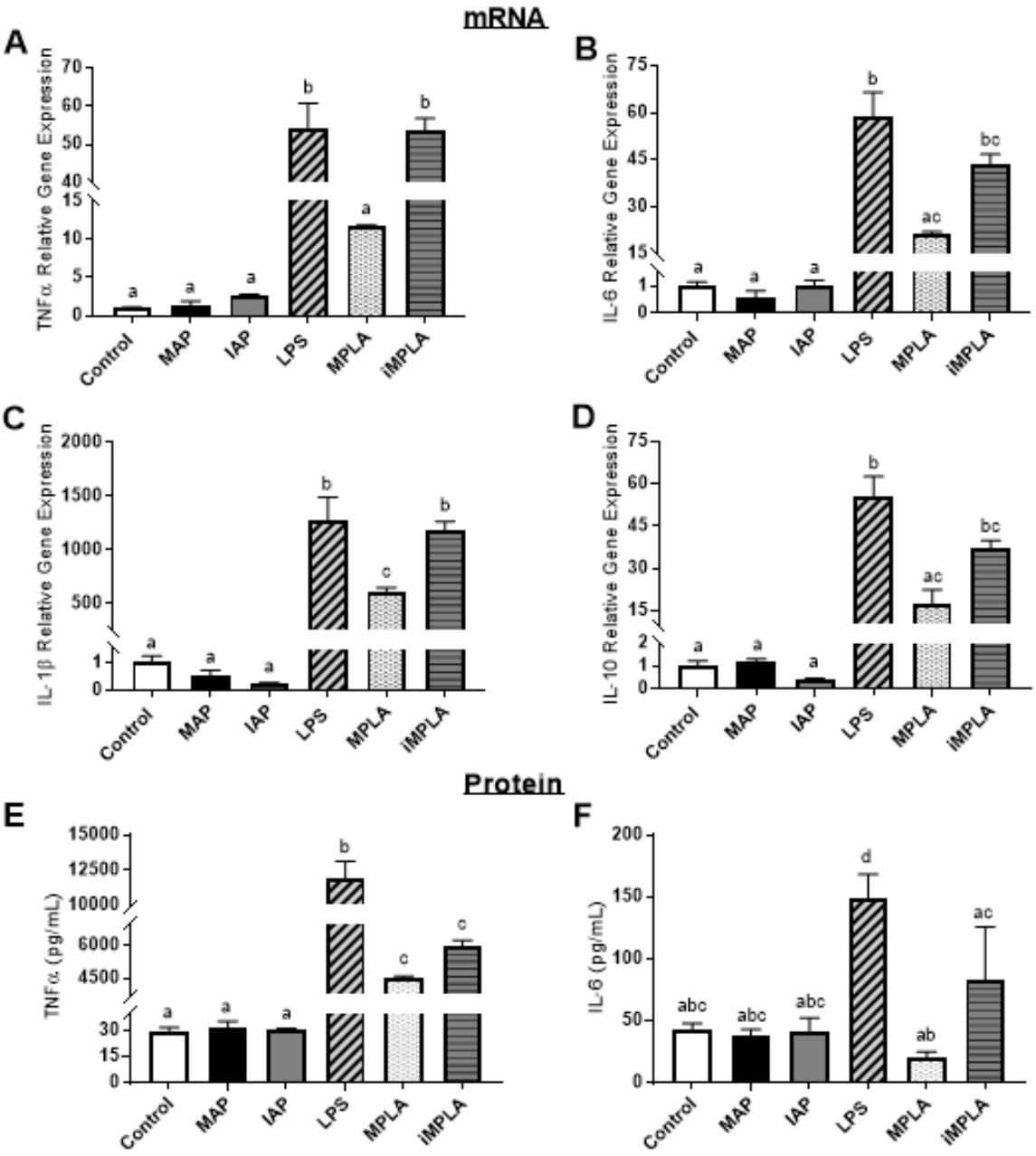


Fig 2.5. Alkaline phosphatase sources differentially detoxify *Salmonella* LPS. RAW264 cells were treated with vehicle, MAP, IAP, *Salmonella* LPS, MAP-detoxified LPS (MPLA) or IAP-detoxified LPS (iMPLA) for 4 hours. (A) TNFα relative gene expression, (B) IL-6 relative gene expression, (C) IL-β relative gene expression, (D) IL-10 relative gene expression. (E) TNFα protein expression. (F) IL-6 protein expression. Treatments with different letters are significantly different. Bars represent the mean ± standard error of the mean (n = 2-6 for gene expression; n = 3 for ELISA)

Again, the above results were confirmed on RAW264 cells using *E.coli* LPS and MPLA derived from *E.coli* LPS **(Fig 2.6a-f)**. There were no differences between control, MAP, or IAP treatment groups for TNFα, IL-6. IL-1β, or IL-10 gene expression in the absence of immune stimulation. *E.coli* LPS increased gene expression for all measured cytokines as compared to control, MAP, and IAP treatment groups ($P<0.001$). *E.coli* iMPLA upregulated TNFα and IL-6 gene expression as compared to MPLA treatment ($P=0.006$; $P=0.014$). There were no differences between iMPLA and MPLA treatments for IL-1β or IL-10 mRNA levels; however, iMPLA had a numerically stronger inflammatory effect. IL-1β gene expression was not different between iMPLA and LPS treatment. Cell culture supernatant from RAW264 cells was subjected to ELISA for determination of TNFα and IL-6 concentrations **(Fig 2.6e-f)**. *E.coli* LPS elevated TNFα protein levels as compared to both MPLA ($P=0.003$) and iMPLA ($P=0.005$); however, there were no differences between dephosphorylated LPS types. IL-6 protein levels were elevated due to *E.coli* LPS treatment as compared to MPLA ($P=0.015$). iMPLA elevated IL-6 secretion similarly to *E.coli* LPS. Together these results indicate that microbe-derived alkaline phosphatase is a more potent detoxifier of both *Salmonella* and *E. coli* LPS than mammalian-derived intestinal alkaline phosphatase.

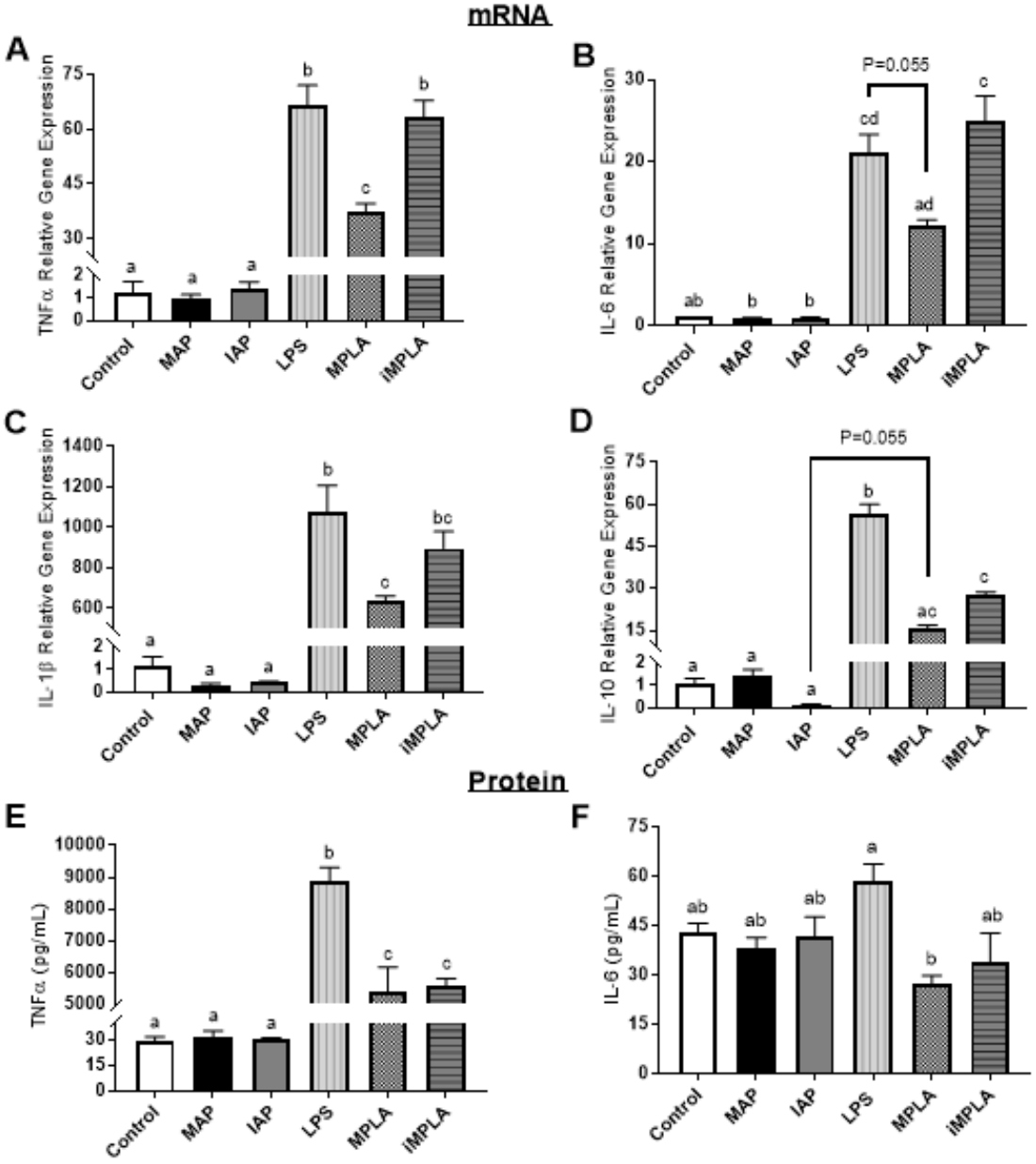


Fig 2.6. Alkaline phosphatase sources differentially detoxify *E. coli* LPS. RAW264 cells were treated with vehicle, MAP, IAP, *E. coli* LPS, MAP-detoxified LPS (MPLA) or IAP-detoxified LPS (iMPLA) for 4 hours. (A) TNFα relative gene expression, (B) IL-6 relative gene expression, (C) IL-β relative gene expression, (D) IL-10 relative gene expression. (E) TNFα protein expression. (F) IL-6 protein expression. Treatments with different letters are significantly different. Bars represent the mean ± standard error of the mean (n = 2-6 for gene expression; n = 3 for ELISA)

To confirm that these results remain across species and in primary cells, the same treatment scheme for both *S. enterica* and *E. coli* LPS was applied to primary pig alveolar macrophages. For all measured cytokines, both *S. enterica* **(Fig 2.7)** and *E. coli* LPS **(Fig 2.8)** treatment significantly elevated cytokine expression ($P<0.001$). Induced gene expression of TNFα, IL-1β, and IL-10 were not different between *Salmonella* LPS and iMPLA treatment groups, and iMPLA treatment significantly elevated gene expression as compared to MPLA treatment ($P<0.001$; $P<0.001$; $P=0.008$) **(Fig 2.7a, c-d)**. There was no difference in treatment-induced IL-6 gene expression between *Salmonella* iMPLA and MPLA **(Fig 2.7b)**. IL-6 protein was quantified, and *Salmonella* LPS, iMPLA, and MPLA elevated the concentration as compared to controls ($P<0.001$) **(Fig 2.7e)**. However, MPLA and iMPLA induced IL-6 protein were not as high as *Salmonella* LPS ($P<0.001$; $P=0.002$) but were not different. Again, the above results were confirmed on porcine alveolar macrophages using LPS and MPLA derived from *E. coli* **(Fig 2.8a-e).** There were no differences in elicited TNFα, IL-6, and IL-10 gene expression between iMPLA and MPLA **(Fig 2.8a-b, d)**. Although iMPLA did not elevate IL-1β gene expression as dramatically as LPS ($P=0.015$), the resultant expression was higher than MPLA treatments ($P<0.001$) **(Fig 2.8c)**. Expectedly, *E. coli* LPS treatment elevated IL-6 protein levels as compared to control treatments ($P<0.001$) and MPLA ($P=0.002$) **(Fig 2.8e)**. There were no differences in IL-6 concentration between MPLA and iMPLA treatment. These results attest to the efficacy of microbial-derived alkaline phosphatase to detoxify LPS as well as, or better than, mammalian-derived IAP. Furthermore, the results are congruent across species and two different LPS types.

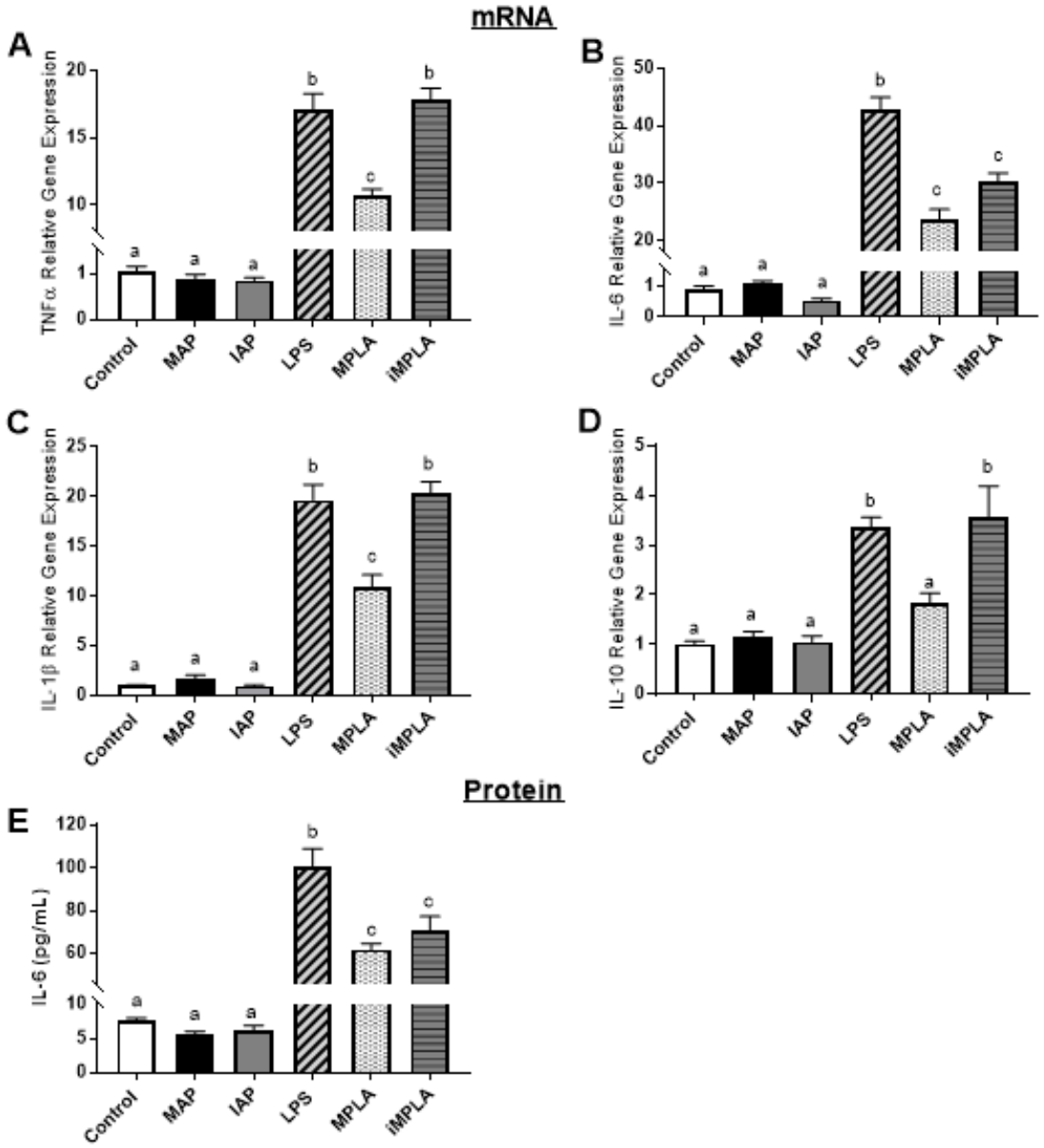


Figure 2.7. MAP-detoxified *Salmonella* LPS elicits lower inflammatory profile in primary macrophages as compared to IAP-detoxified LPS. Gene expression of primary cells 4 hours post treatment of vehicle, MAP, IAP, *Salmonella* LPS, MPLA, or iMPLA. Treatment with MPLA elicited lower levels of pro-inflammatory cytokines than iMPLA for TNFα (A), IL-1β (C), and IL-10 (D). There was no difference between MPLA and iMPLA for IL-6 gene expression (B) or IL-6 protein expression (E). Data are presented as means ± SEM (n = 5-11). Treatment means with different letters are significantly different.

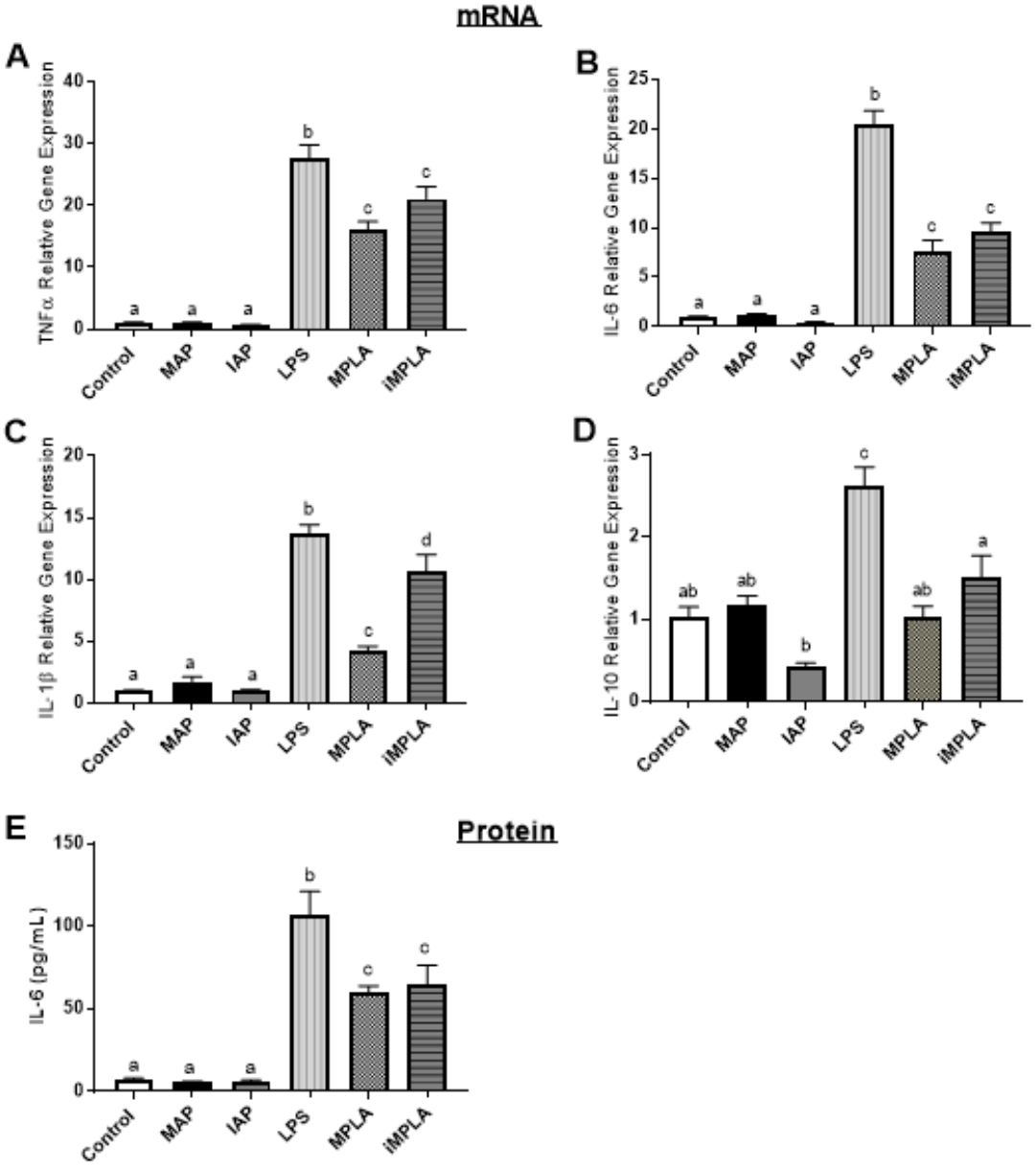


Figure 2.8. Gene and protein expression profiles of primary alveolar macrophages stimulated with detoxified *E.coli* LPS vary between alkaline phosphatase sources. Primary pig alveoalar macrophages were treated with vehicle, MAP, IAP, *E.coli* LPS, MPLA, or iMPLA for 4 hours and gene expression of TNFα (A), IL-6 (B), IL-1β (C), IL-10 (D) and protein expression of IL-6 (E) quantified. Data are presented as means ± SEM (n = 5-11). Treatment means with different letters are significantly different.

Microbial-derived alkaline phosphatase detoxified *Salmonella* LPS decreased the inflammatory response and altered sickness behavior in weaning piglets

To determine the ability of MPLA to reduce *Salmonella* LPS bioactivity *in vivo*, piglets were injected i.p. with saline, LPS, or MPLA. (**Fig 2.9a-d).** At 2 and 4 hours post-treatment, serum levels of TNFα were higher in LPS-treated pigs than saline piglets (P=0.005; P=0.033, respectively), while circulating TNFα was not affected in MPLA-treated pigs. (P=0.419; P=0.093, respectively). MPLA and LPS were not different at any time point. There was a main effect of treatment with LPS injected piglets (P<0.001) and MPLA injected piglets (P=0.02) having higher TNFα serum levels than saline injected piglets. There were no main effects between LPS or MPLA. IL-6 serum levels were elevated at 2 hours post-injection by LPS treatment (P=0.005), but MPLA treatment did not elevated IL-6 levels (P=0.112). LPS treatment affected cytokine production of TNFα and IL-6 in a time-dependent manner and MPLA treatment did not, demonstrating the modulated inflammatory response. IL-6 serum levels had no main effects of treatment and there were no interactions between time and treatment for serum levels of anti-inflammatory cytokines IL-10 and IL-4. However, there were main effects of treatment with LPS raising circulating IL-10 levels above control (P=0.025) and MPLA piglets (P=0.006). IL-4 levels were different between treatments with LPS treatment elevating serum levels above both control (P=0.045) and MPLA (P=0.05) treated piglets.

Since the liver and spleen play a prominent role in the removal of LPS from systemic circulation and are important for the acute phase response, gene expression of pro- and anti-inflammatory cytokines were measured. In the liver, LPS raised TNFα, IL-6, IL-1β, and IL-10 mRNA higher than MPLA treatment but was not significantly different (**Fig 2.9e).** Also, TNFα was upregulated in the spleen following LPS treatment as compared to control pigs and MPLA

pigs (P=0.006; P=0.029, respectively) (**Fig 2.9f)**. LPS treatment also raised splenic IL-6, IL-1β, and IL-10 mRNA levels, however, levels were not different from MPLA treatment.

Behavior was recorded throughout the 4 hours for each cohort to determine if MPLA elicited sickness behavior that is well documented for LPS. Total time spent eating and being active was calculated per pig and are presented as an average across all cohorts. Throughout the 4 hour treatment period, piglets injected with LPS spent less time eating than control or MPLA groups (P=0.031) (**Fig 2.10a)**. There was a decrease in activity level between treatment groups, with control piglets being the most active and LPS piglets being the least active **(Fig 2.10b).** These results demonstrate that MAP-detoxified LPS mitigates sickness behavior.

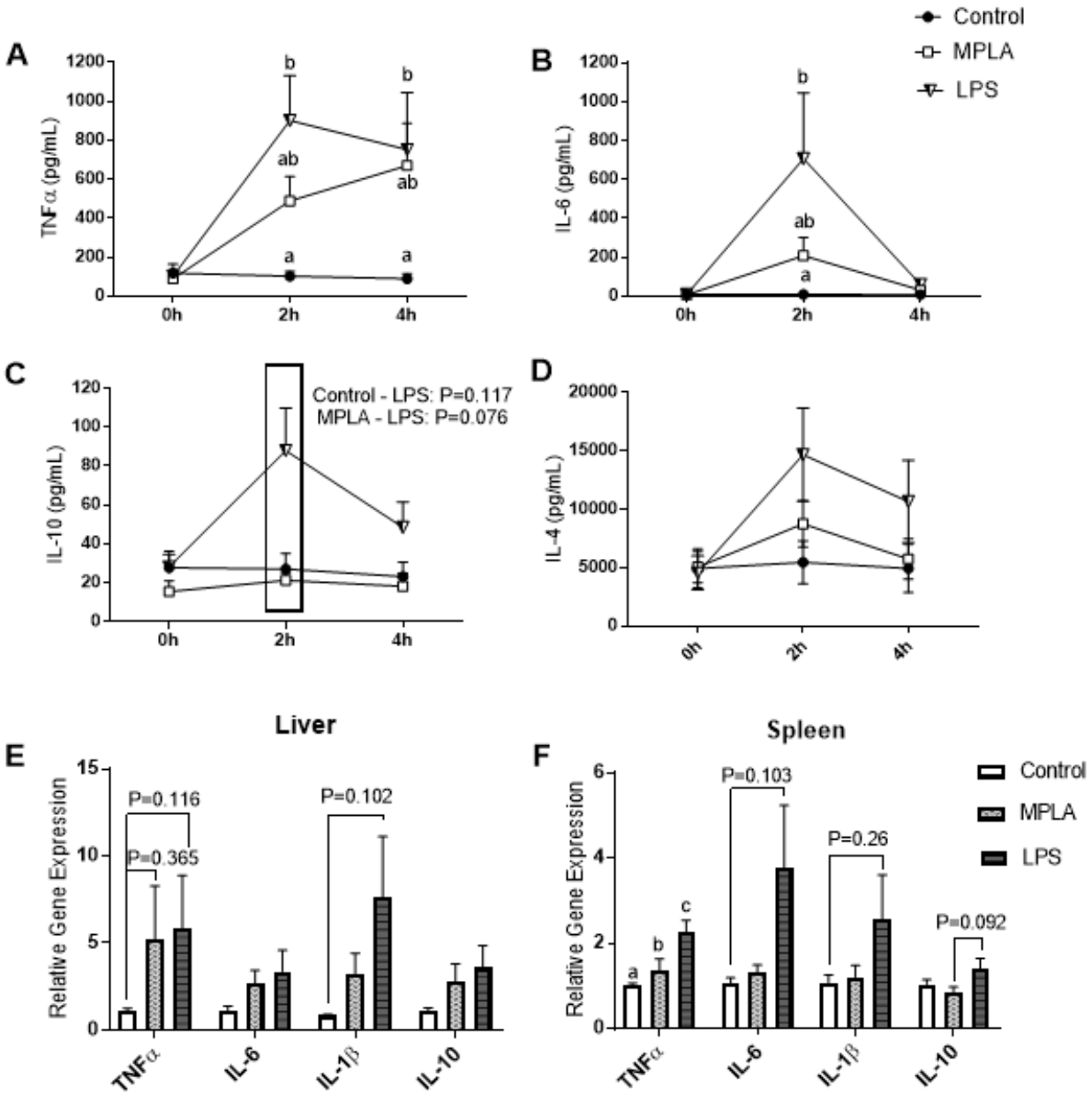


Figure 2.9. LPS and MPLA-induced circulating cytokines levels in weaning piglets at 0, 2, and 4 hours post i.p. injection and liver and spleen cytokine gene expression. LPS and MPLA were injected i.p. at 10 ug LPS/kg bw and control piglets recieved same volume of vehicle and serum collected for cytokine profile of TNFα **(A)**, IL-6 **(B)**, IL-10 **(C)**, and IL-4 **(D).** Gene expression for TNFα, IL-6, IL-1β, and IL-10 at 4 hours post-injection for liver **(E)** and spleen **(F).** Data are presented as means ± SEM (n = 4-6 per time point). Treatment means with different letters are signicantly different.

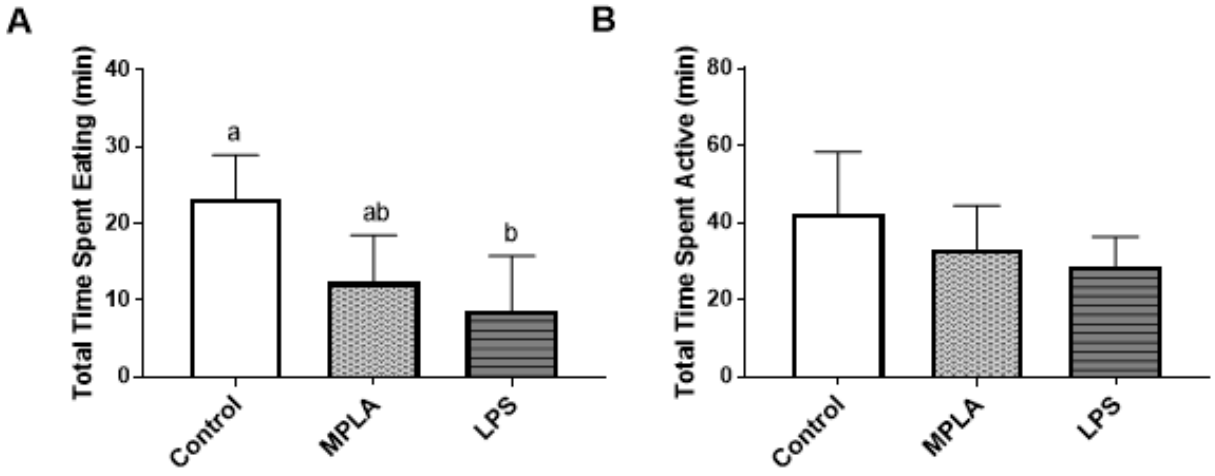


Figure 2.10. Microbial-derived AP-detoxified LPS produces modified sickness behavior in weaning piglets. Piglets injected with vehicle, LPS, or MPLA were recorded for 172 minutes and instantanous scan sampling utilized in 1 minute intervals to record total time spent eating and being active. LPS significantly decreased feed intake as compared to control (P=0.031), while MPLA-treated piglets had no decline **(A)**. There were no significant differences in time spent being active **(B).** Data are presented as means ± SEM (n = 6). Treatment means with different letters are significantly different.

2.5 Discussion and Future Directions

Lipopolysaccharide (LPS), a Gram-negative bacteria cell component, has long been implicated in systemic inflammation and septic shock. It is well known that the toxicity of LPS is highly dependent upon its phosphorylation state, and that removal of one of the two phosphate groups from the lipid A moiety results in significantly lower toxicity[97]. In the present study, we found that pre-incubation of LPS with microbe-derived alkaline phosphatase (MAP) reduced the toxicity of *Salmonella* and *E. coli* LPS *in vitro* and *in vivo.* Furthermore, novel findings indicate that MAP has an increased ability to dephosphorylate LPS, as compared to intestinal alkaline phosphatase.

Secretion of cytokines is necessary for activation of the immune system and migration of immune cells to sites of infection. However, LPS can overstimulate TLR4 receptors and lead to downstream overexpression of pro-inflammatory cytokines that are detrimental to the host. As macrophages are part of the second line of defense in the body, RAW264 and primary pig alveolar macrophages were used to study the capacity of MAP to detoxify LPS *in vitro.* Overproduction of pro-inflammatory cytokines has deleterious effects on homeostatic balance and can be lethal. LPS-induced TNFα has long been implicated to be a key mediator in septic shock and is necessary and sufficient for liver failure and cytotoxicity[46,48,98]. An increase in IL-6, IL-1β, and IL-10 levels have also been associated with chronic inflammation and sepsis[99]. MAP-dephosphorylated *Salmonell*a and *E. coli* LPS were unable to elicit the same robust inflammatory response as native LPS in RAW264 cells or primary alveolar porcine macrophages, as demonstrated by reduced pro- and anti-inflammatory cytokine output. These results support Polestra *et al.*(1997), who showed that LPS is a substrate for AP and suggested AP has a role in endotoxin dephosphorylation[73,74]. Furthermore, these results match with Koyama *et al.* (2002),

who showed that LPS toxicity on endothelial cells was diminished *in vitro* when LPS was pretreated with IAP. Interestingly, MAP-detoxified *Salmonella* and *E. coli* LPS decreased gene expression of IL-10 to control treatment levels in RAW264 macrophages. MAP-detoxified *E. coli* LPS lowered IL-10 gene expression to control levels in primary pig alveolar macrophages. Produced by monocytes, lymphocytes, and macrophages, IL-10 is an anti-inflammatory cytokine that has been reported to inhibit the expression of pro-inflammatory cytokines TNFα, IL-6, and IL-1β[100,101]. Our results indicate that the lower levels of elicited pro-inflammatory cytokines from the MPLA treatments did not require induction of high levels of IL-10 to modulate the acute inflammatory response. *E. coli* and *Salmonella* LPS treatment alone induced higher levels of pro-inflammatory cytokines and thus needed to transcribe IL-10 to, unsuccessfully, inhibit cytokine production. These results attest to the ability of MAP to detoxify LPS and modulate the inflammatory response. The extent to which this occurs and further effects of this would require further research.

The physiological function of mammalian intestinal alkaline phosphatase is to detoxify LPS at the brush border and protect systemic circulation from an antigenic breach. This study aimed to compare the efficacy of MAP to reduce the LPS-induced immune response, with the ability of IAP to detoxify LPS *in vitro*. Novel results indicate that MAP has more potent LPS detoxification properties than IAP. MAP-detoxified *Salmonella* LPS was less inflammatory than IAP-detoxified LPS as shown by significantly lower TNFα and IL-1β gene expression in RAW264 cells and lowered gene expression of TNFα, IL-1β, and IL-10 in primary alveolar porcine macrophages. Not only did MPLA evoke lower levels of pro- and anti-inflammatory cytokines, but IAP-detoxified *Salmonella* LPS elicited TNFα and IL-6 gene expression and IL-6 protein expression that was not different from *Salmonella* LPS treatments. Similarly, treating

RAW264 cells with MAP-detoxified *E. coli* LPS elicited significantly lower levels of TNFα and IL-6 mRNA than IAP-detoxified *E. coli* LPS, while iMPLA was not different from LPS for TNFα, IL-6, or IL-1β expression. These results indicate that pre-incubation of LPS with one of the two alkaline phosphatases, at the same ratio of AP:LPS results in MPLA being significantly less inflammatory and iMPLA being either more inflammatory than MPLA or just as toxic as LPS treatments alone. Future studies should focus on the specifics of enzymatic activity for the two AP and determine any differences in the kinematics of LPS dephosphorylating ability.

Weaning piglets were challenged with vehicle, *Salmonella* LPS, or MAP-detoxified *Salmonella* LPS to determine if the *in vitro* results could be recapitulated *in vivo*. The authors chose to use *Salmonella* LPS for the immune challenge due to *in vitro* results showing that detoxified *Salmonella* LPS was less stimulatory than detoxified *E. coli* LPS. *Salmonella* LPS significantly raised circulating TNFα levels at two and four hours post-injection, while MPLA-treated piglets were never different than controls. These results agree with Bentala *et al.* (2002) in which Balb/c mice were i.p. injected with *Salmonella minnesota* LPS or MPLA from *S. minnesota.* Bentala and coworkers reported that MPLA treatment was not sufficient to elevate circulating TNFα at one, two, three, or four hours post injection[102]. *Salmonella* LPS also significantly upregulated plasma IL-6 levels at two hours post-injection, in contrast, MPLA treatment did not affect circulating IL-6. IL-10 is released to inhibit the production of TNFα and IL-16 and is associated with the disappearance of clinical signs and return to homeostasis. LPS treatment significantly increased circulating IL-10 above both saline and MPLA piglets. There was no differences between saline or MPLA-injected piglets for circulating IL-10, demonstrating the modulated immune response. Finally, IL-4 plasma levels were significantly different than LPS and control treated piglets, and LPS and MPLA treated piglets. IL-4 is an essential anti-

inflammatory cytokine and activator of T_h2 helper cells, which secrete more IL-4 in a positive feedback manner. The elicitation of IL-4 by LPS but not MPLA, confirms that MPLA is significantly less toxic to a weaning piglet and therefore requires less immune activation. Furthermore, IL-4 signaling activates STAT6, which mediates repression of PPARα by preventing PPARα recruitment to target genes. This leads to a significant reduction in the expression of proteins involved in the citric acid cycle, fatty acid oxidation, and oxidative phosphorylation[103,104]. This release of glucose and fatty acids is used to fuel the activated immune system; however, glucose and fatty acids are used for growth and development in a young mammal. Therefore, these results indicate the LPS-induced increase in circulating IL-4 levels during the immune challenge could inhibit muscle accretion and bone growth by releasing those building blocks, but MAP-detoxified LPS would not interfere with this process. More research is needed to determine if exogenous MAP supplementation would interact with LPS in the intestinal lumen to produce MPLA, and MAP would exert a protective effect overgrowth arrest during weaning.

Cytokine gene expression in the liver and spleen were examined at the end of the 4-hour immune challenge to confirm ELISA results. Splenic TNFα expression was significantly elevated in LPS injected piglets but not with MPLA. LPS also elevated IL-6, IL-1β, and IL-10 levels in the liver and spleen. TNFα is promptly released from immune cells and is a potent activator of the NF-κB pathway, which amplifies the immune response through increasing pro-inflammatory cytokine expression[98]. The inability of MPLA to elicit a change in splenic TNFα gene expression attests to the ability of MAP to detoxify LPS.

Given that MPLA is derived from LPS, and *in vitro* results showed a significant decrease in TLR4 stimulation, we hypothesized that i.p. administration of MPLA would result in

decreased piglet sickness behavior. Behavior was examined throughout the study for activity and food intake, and with feed being removed 3 hours before the study to control for natural lack of appetite. LPS-treated piglets spent significantly less time eating and were less active than vehicle or MPLA-injected piglets. This is in contrast to Eimerbrink *et al.* (2016) that showed MPLA stimulation alone disrupted burrowing behavior in C57BL/6J mice, as compared to saline-treated mice[105]. This difference in results could be attributed to species differences and sickness behavior paradigms. The same study showed pretreatment with MPLA protected against LPS-induced sickness behavior. Endotoxin tolerance is the reduced capacity of a cell to respond to LPS after initial exposure and occurs as the result of persistent TLR4 stimulation[106,107]. Contemporary literature is exploring the ability of TLR4 agonists, such as MPLA, inducing cross-tolerance to LPS; however, the ability of microbial AP-derived MPLA to exert these protective effects has yet to be examined and could be a therapeutic agent for LPS-mediated diseases.

In a therapeutic context, work on the beneficial effects of exogenous MPLA and IAP supplementation have been wide-reaching. TLR4 activation and the subsequent pro-inflammatory response can detrimental to healthy growth and function, although the immune stimulatory properties play a significant role in host protection. MPLA is currently being utilized as a vaccine adjuvant for its ability to stimulate the immune system, without overwhelming host defenses[108]. TLR4 stimulation with MPLA also significantly improves Alzheimer's disease-related pathology by increasing the ability of microglia to phagocytize Aβ plaques[109]. Oral supplementation of IAP decreases the severity of *Clostridium difficile* infection and protects against antibiotic-induced dysbiosis[110]. Finally, exogenous IAP has been shown to prevent high

fat diet-induced metabolic syndrome and reverse changes associated with metabolic syndrome[111].

In this paper, we have shown that microbial-derived alkaline phosphatase detoxifies both *Salmonella* and *E. coli* LPS in murine macrophages and primary porcine alveolar macrophages, and i.p. administration of the product MPLA in weaning piglets resulted in significantly lower circulating pro- and anti-inflammatory cytokines, lower mRNA expression of cytokines in primary immune organs and is not potent enough to elicit sickness behavior. Novel findings indicate a bolstered ability of MAP to detoxify Gram-negative bacteria, as compared to IAP. Taken together, these present findings suggest microbial-derived AP, and MPLA could represent a safe and effective therapeutic approach to LPS-mediated disease as both prevention and treatment.

CHAPTER 3: DIETARY INTERVENTION WITH MICROBIAL-DERIVED ALKALINE PHOSPHATASE EXERTS PROTECTIVE EFFECTS AGAINST POST-WEAN SYNDROME IN PIGLETS

3.1 Abstract

Piglet weaning is associated with growth plateaus, inflammation, and increased endotoxin load in the gastrointestinal tract (GIT). Furthermore, weaning is characterized by perturbations in GIT enzymatic activity and morphological changes, which drive further inflammation and decrease the digestive and absorptive capacity of the small intestine, as well as overwhelm the innate immune response. Increased inflammation and bacterial load lead to displaced tight junction proteins (TJP) and inhibits the immunoprotective effects of intestinal alkaline phosphatase (IAP). Recent *in vivo* data from our lab has shown that microbial-derived alkaline phosphatase (MAP) is capable of detoxifying lipopolysaccharide (LPS), an endotoxin from Gram-negative bacteria such as *E. coli*. In the present study we focused on elucidating the effects of exogenous MAP supplementation on piglets post-wean. We found that MAP-fed piglets had a higher average daily gain (P=0.004) and increased villus height to crypt depth ratio in the duodenum, jejunum, and ileum (P<0.001). Compared to control piglets, MAP-fed piglets had increased gene expression of TJP *ZO-1* in the jejunum (P<0.001) and ileum (P=0.05), and decreased gene expression of duodenal and jejunal *claudin-1* (P=0.04; P=0.02). Duodenal (P=0.009), jejunal (P=0.006) and ileal (P=0.003) *IAP* gene expression was substantially upregulated in MAP-supplemented piglets. Finally, exogenous MAP increased alkaline phosphatase enzymatic activity in the digesta of the duodenum (P<0.001), jejunum (P=0.03) and ileum (P=0.002); and the mucosa of the duodenum (P=0.04), jejunum (P=0.002), and ileum

(P=0.02). Taken together, these results indicate a novel therapeutic intervention approach to protect piglets against weaning-induced growth plateaus, disruptions against GIT tight junction proteins, and inhibition of IAP and AP activity due to increased bacterial load and inflammation during the post-weaning period.

3.2 Introduction.

In the modern industrial swine industry, weaning is the most challenging and stressful point in the pig's life due to major nutritional, psychological, and environmental stressors. The post-weaning phase is often accompanied by reduced growth performance, reduced feed intake, and increased incidence of diarrhea and bacterial translocation[1,2]. The gastrointestinal tract is lined with a single layer of epithelial cells that form a selective barrier and act as the first line of defense against potentially harmful compounds and microorganisms in the intestinal lumen. Intestinal barrier dysfunction is characterized by increased intestinal permeability, or "leaky gut," which allows harmful immunogenic agents to cross the epithelium and gain access to protected tissues and systemic circulation. This breach of the epithelial lining and subsequent translocation of luminal contents contributes to increased inflammation, malabsorption, diarrhea, and potential enteric disease[4,19,20].

Enterocytes are joined together by tight junctions that consist of proteins which function to connect the cytoskeletons of adjacent enterocytes[24]. Tight junctions are mainly constructed from the transmembrane protein complexes occludin and claudin, and the cytosolic protein zonula occluden. Alterations in tight junction proteins (TJP) are biomarkers of increased intestinal permeability. Increased translocation of enteric pathogens, including endotoxins from Gram-negative bacteria such as *E. coli*, disrupt these proteins and increase intestinal permeability[25]. Endotoxins, such as Gram-negative bacteria cell wall component lipopolysaccharide (LPS), spontaneously release and can cause structural changes in the small intestine by disrupting the structure of tight junctions and causing the internalization of TJP occludin, which allows pathogens to cross into systemic circulation[61,64]. Inflammatory mediators, such as the pro-inflammatory cytokine TNFα, are released from cells, such as innate lymphoid

cells and epithelial cells, following LPS-stimulation and increase intestinal permeability by disrupting expression of claudins and occludins and by altering the lipid environment of phospholipid membranes to displace TJP[65,66].

The epithelial lining of the small intestine is covered in villi, small finger-like projections, to increase surface area for digestion and absorption[5]. Optimal function of the small intestine favors long villi. Following weaning, there is a brief period of villous atrophy and crypt hyperplasia that leads to impaired gut absorptive capacity and decreases digestive and brush-border enzymatic activity[3,7,8].

Alkaline phosphatases (AP) are homodimeric enzymes that catalyze the hydrolysis of monoesters of phosphoric acid and transphosphorylation reactions. Intestinal alkaline phosphatase (IAP) is a glycoprotein anchored in the apical membrane of the small intestine and is found in the highest levels in the duodenum, followed by the jejunum and ileum. IAP has several biological roles, including being a negative regulator of intestinal fat absorption, maintaining bicarbonate secretion and pH balance, and exerting immuno-protective effects[68,69]. The toxicity of LPS, specifically the lipid A moiety, is dependent upon its phosphorylation state[72]. A significant role of AP *in vivo* is to detoxify bacterial LPS that is present in the intestinal lumen. High levels of IAP can control LPS-induced inflammation in two ways: by dephosphorylating LPS and decreasing TLR4 stimulation, and by preventing NF-κB translocation to the nucleus by inhibiting the phosphorylation of two critical proteins in the pathway, IκBα and RelA/p65[75]. The ability of IAP to dephosphorylate LPS, at physiological pH, and to reduce the toxic effects *in vivo* was first shown in 1997[73,74].

During weaning, piglets go through a fasting period as their immature digestive and immune systems are catching up, and it is well known that fasting dramatically decreases IAP activity[78].

Porcine epidermal growth factor (pEGF) is found in sow milk and contributes to postnatal gut mucosal growth and development, and a recent study showed that supplementing weaned pigs with exogenous pEGF caused an increase in gene expression and protein activity of digestive enzymes in the GIT, including IAP[81,82]. Fasting also decreases *Lactobacillus* populations, which allow for overpopulation of Gram-negative bacteria and increased LPS concentrations in the lumen, and the probiotic *Lactobacillus casei* has been shown to stimulate IAP activity[80]. IAP's ability to reduce bacterial translocation and attune commensal microbiota may be an ancillary effect of pH regulation, as an alkaline microenvironment is unfavorable for the growth of pathogens[68].

Previous work by members of our lab showed that, like AP, microbial-derived AP (MAP) can detoxify Gram-negative bacterial LPS from *E. coli* and *Salmonella* and reduce the LPS-mediated inflammatory effects *in vitro* and *in vivo*. The objectives of this study were to determine if exogenously supplemented microbial-derived AP would increase AP activity in the small intestine and exert a protective effect over post-weaning syndrome. We hypothesized that feeding post-weaning piglets MAP would increase duodenal, jejunal, and ileal AP activity and protect against the weaning-induced decrease in IAP gene expression. Furthermore, we quantified the effects of exogenous MAP supplementation on tight junction proteins in the small intestine and determined the protective effects on villus height and crypt depth.

3.3 Materials and Methods

Animals and Diets

Sixteen male PIC piglets from University of Illinois swine herd were naturally farrowed and weaned at 21 days of age. Piglets were kept on the premises of University of Illinois Imported Swine Research Farm and randomly divided into two treatment groups (n=8) controlling for litter of origin and body weight. Piglets were housed in four pens of four piglets each. All four pens were kept on standard phase 1 diet for one-week post-weaning to acclimate to the novel environment and piglets **(Table 3.1)**. On postnatal day 28, two pens were switched to a standard phase 2 diet, and two pens were placed on a standard phase 2 diet supplemented with 4,000 IU/kg of MAP **(Table 3.1)**. The experimental and control diets and fresh, clean drinking water were offered *ad libitum* throughout the experimental period. All piglets were maintained on control or experimental diet until postnatal day 42. Postnatal day 42, piglets were euthanized according to University of Illinois swine farm standard operating procedure as described in the AVMA Guidelines on Euthanasia, and tissues were collected for analysis. Representative segments from the duodenum, jejunum, and ileum were collected for histology. Digesta contents and mucosa were collected from duodenum, jejunum, and ileum and flash frozen for gene expression and AP activity. Weight of piglets was determined at post-natal day 21, 28, 35, and 42. Group housing and *ad libitum* feed prevented individual feed intake data collection. All procedures were approved by the University of Illinois at Urbana-Champaign Institutional Animal Care and Use Committee.

Table 3.1 Composition of experimental diets

Ingredient (%)	Control	MAP
Corn	42.02	42.01
Soybean meal	31.50	31.49
Whey	20.00	19.99
Fat	3.00	3.00
Dicalcium phosphate	0.70	0.70
Limestone	1.20	1.20
L-lysine-HCl	0.35	0.35
DL-methionine	0.15	0.15
L-threonine	0.08	0.08
Vitamin premix	0.20	0.20
Swine trace mineral premix	0.35	0.35
Salt-white	0.20	0.20
Zinc oxide	0.25	0.25
Microbial alkaline phosphatase (MAP)	0.00	0.03
Total	100.00	100.00

Alkaline Phosphatase Activity

AP activity was determined using a colorimetric assay kit (Abcam, USA, ab83369) according to the manufacturer's protocol. Briefly, digesta was collected from the duodenum, jejunum, and ileum and suspended in an equal volume of 0.9% saline plus protease inhibitor cocktail (11873580001; Sigma-Aldrich) at pH 8.5, centrifuged and supernatant collected for assay. Mucosal scrapings were collected with a glass slide from the duodenum, jejunum, and ileum and homogenized with equal volume 0.9% saline plus protease inhibitor cocktail at pH 8.5. The homogenized mucosa was centrifuged and pelleted and the supernatant collected for assay. AP activity was determined with respect to the release of *p*-nitrophenol from the p-nitrophenylphosphate (*p*NP) substrate. Each reaction was initiated by the addition of *p*NP to small intestine mucosa and digesta, and the reaction was stopped sixty minutes later with the addition of stop solution. Optical density was measured at 405 nm to quantify the amount of *p*-

nitrophenol produced. AP activity was determined using the below equation where B equals the amount of *p*NP in the sample well calculated from the standard curve, ΔT is the reaction time in minutes, V is the original sample volume in the reaction well, and D is the sample dilution factor:

$$ALP\ Activity = \left(\frac{B}{\Delta T * V}\right) * D$$

Gene Expression

Total RNA from duodenum, jejunum, and ileum mucosal scrapings were isolated using the Tri Reagent protocol (Invitrogen). Complimentary DNA was synthesized using a high-capacity RT kit (Applied Biosystems, Grand Island, NY, USA) according to the manufacturer's instructions. Quantitative real-time PCR was performed using the Applied Biosystems Taqman Gene Expression Assay protocol, and relative gene expression was determined from the fluorescent data on an ABI PRISM 7900HT- sequence detection system (PerkinElmer, Waltham, MA). All primers and probes for quantitative PCR were obtained from Integrated DNA Technologies (Coralville, IA, USA). Genes of interest were tight junction proteins claudin 1 (NM_001244539.1), ZO-1 (XM_003480423.4), occludin (NM_001163647.2), and intestinal alkaline phosphatase (XM_003133729.4). Mucosal mRNA abundance was compared to reference gene RPL19 (Ss03375624_g1). PCR conditions included 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds, and 1 cycle at 60°C for 1 minute. Gene expression was determined using the $2^{-\Delta\Delta Ct}$ calculation method as previously described [96]. Data are expressed as fold change versus controls.

Histology

Representative sections of the duodenum, jejunum, and ileum were removed at places along the whole length of the small intestine. The samples were fixed in 10% neutral buffered formalin for 48 hours and then embedded in paraffin wax by standard techniques. From each sample, five transverse sections were fixed on two slides and stained with hematoxylin and eosin. To reduce variability in measuring, villus height and crypt depth were measured by the same person who was blinded to treatment. All morphological measurements were made using the Nanozoomer-XL (Hamamatsu Photonics, Bridgewater, NJ). Villi height were measured by picking five intact, representative villi for each small intestine section across two slides per S.I. section. The height of each villus was measured from tip to crypt-villus junction. Crypt depth was measured from five crypts for each small intestine section that was associated with intact villi. Crypt depth was measured from the crypt-villus junction to the base.

Statistics

Data were analyzed by one-tail unpaired students T-test with Welch's correction when appropriate or by Mann-Whitney U-test. Statistical significance was set at $p \leq 0.05$. Analysis was performed using GraphPad Prism version 7.00 for Windows (GraphPad Software, La Jolla, CA) or SAS™ 9.4 software (SAS Institute, Cary, NC, USA). All data are expressed as means ± standard error of the mean.

3.4 Results

Feeding weaned piglets microbial-derived alkaline phosphatase mitigates weaning-induced growth plateau

Piglets were weaned, and both treatment groups received a standard phase 1 diet for one-week post-weaning to normalize the gastrointestinal tract and then given a standard phase 2 diet for two weeks with or without MAP supplementation **(Fig 3.1a).** Piglets supplemented with 4,000 IU/kg feed MAP had a heavier body weight after 14-days of MAP supplementation than piglets on a standard phase 2 diet (P=0.016) **(Fig 3.1b)**. Furthermore, piglets fed the MAP-supplemented diet had a higher average daily gain during the second week of the experimental period (P=0.004). **(Fig 3.1c)**.

MAP supplementation exerts positive effects on villus height and crypt depth

Piglets receiving a phase 2 diet supplemented with MAP had longer villi in the duodenum (P=0.006) and jejunum (P=0.03) than control fed piglets **(Table 3.2)**. There were no differences (P=0.XX) in ileal villus height. Furthermore, MAP fed piglets had more shallow crypt depth in the duodenum (P=0.002), jejunum (P=0.002), and ileum (P<0.001). Finally, villus height to crypt depth ratio was calculated and MAP fed piglets had an increased VH:CD ratio in the duodenum, jejunum, and ileum (P<0.001). Differences in VH and CD are visible in representative sections of the duodenum, jejunum, and ileum for control and MAP fed piglets **(Fig 3.2).**

Piglets supplemented with MAP post-wean have increased small intestine alkaline phosphatase activity

To determine if exogenous MAP supplementation could be detected in the small intestine, digesta and mucosa from duodenum, jejunum, and ileum were collected, and the amount of *p*NP hydrolyzed quantified. We first investigated if MAP activity could be detected in the small intestine and would not be hydrolyzed by stomach acid **(Fig 3.3a-c).** Compared to control weaning piglets, piglets fed a phase 2 diet supplemented with MAP had substantially increased AP activity in the digesta of the duodenum ($P<0.001$), jejunum ($P=0.03$) and ileum ($P=0.002$). Furthermore, we determined an increase in mucosal AP activity in the duodenum ($P=0.04$), jejunum ($P=0.002$), and ileum ($P=0.02$) of MAP-supplemented piglets compared to animals fed the control diet **(Fig 3.3d-f).** Taken together, the above results indicate that feeding exogenous MAP during weaning rescues weaning-induced decreases in AP enzymatic activity in small intestinal mucosa and digesta.

MAP-supplemented piglets are protected against weaning-induced downregulation of intestinal alkaline phosphatase and tight junction protein gene expression.

Since IAP levels are known to decrease during weaning, we sought to determine the effects of feeding MAP post-weaning on IAP gene expression in the small intestine **(Fig 3.4a)**. Piglets fed the experimental diet were substantially protected against weaning-induced downregulation of IAP in the duodenum ($P=0.009$), jejunum ($P=0.006$), and ileum ($P=0.003$). Tight junction proteins in the gastrointestinal tract are disturbed during weaning and contribute to increased gut permeability. Therefore, we measured ZO-1, claudin-1, and occludin gene expression in the duodenum, jejunum, and ileum **(Fig 3.4b-d)**. Compared to control piglets,

MAP-fed piglets had higher levels of ZO-1 mRNA in the jejunum ($P<0.001$) and ileum ($P=0.05$). MAP-supplemented piglets had a decrease in duodenal ($P=0.04$) and jejunal ($P=0.02$) claudin-1 gene expression. There were no differences between control and MAP-supplemented piglets for occludin gene expression in the small intestine. Taken together, these results indicate that supplementing piglets with MAP during the post-weaning phase protects against weaning-induced decreases in IAP gene expression and disrupted tight junction proteins.

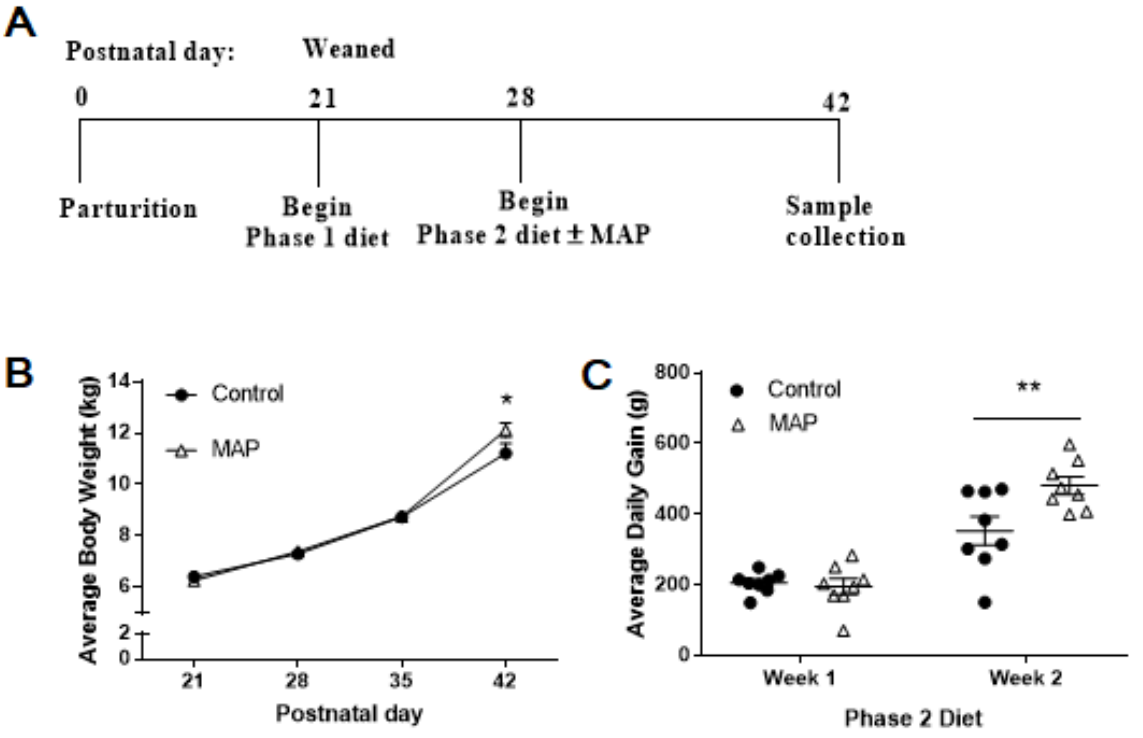


Figure 3.1. MAP-supplemented piglets are protected from growth arrest during weaning. Piglets were weaned at postnatal day 21 and maintained on standard phase 1 diet to normalize GIT. On postnatal day 28 piglets were given standard phase 2 diet with or without 4,000 IU/kg feed MAP **(A).** Piglets supplemented with MAP gained significantly more mass during the second week of phase 2 diet (P=0.016) **(B).** Average daily gain was increased during phase 2 diet feeding supplemented with MAP **(C).** Data are represented as mean ± SEM (n = 8).

Table 3.2 Effects of MAP supplementation on villus height (VH), crypt depth (CD), and villus height: crypt depth ratio (VH:CD) of piglets three weeks post-wean

Effects	Diet		*P-value*
	Control	MAP Supplemented	
VH (µm)			
Duodenum	451 ± 20.3	560 ± 31.4	0.006
Jejunum	452 ± 45.3	553 ± 20.1	0.030
Ileum	340 ± 25.4	383 ± 22.8	0.115
CD (µm)			
Duodenum	206 ± 8.3	161 ± 10.3	0.002
Jejunum	199 ± 11.3	148 ± 8.4	0.002
Ileum	175 ± 2.5	112 ± 5.1	<0.001
VH:CD ratio			
Duodenum	2.22 ± 0.13	3.51 ± 0.09	<0.001
Jejunum	2.29 ± 0.19	3.59 ± 0.07	<0.001
Ileum	1.94 ± 0.14	3.44 ± 0.20	<0.001

(n = 8) The results are presented as mean ± SEM.

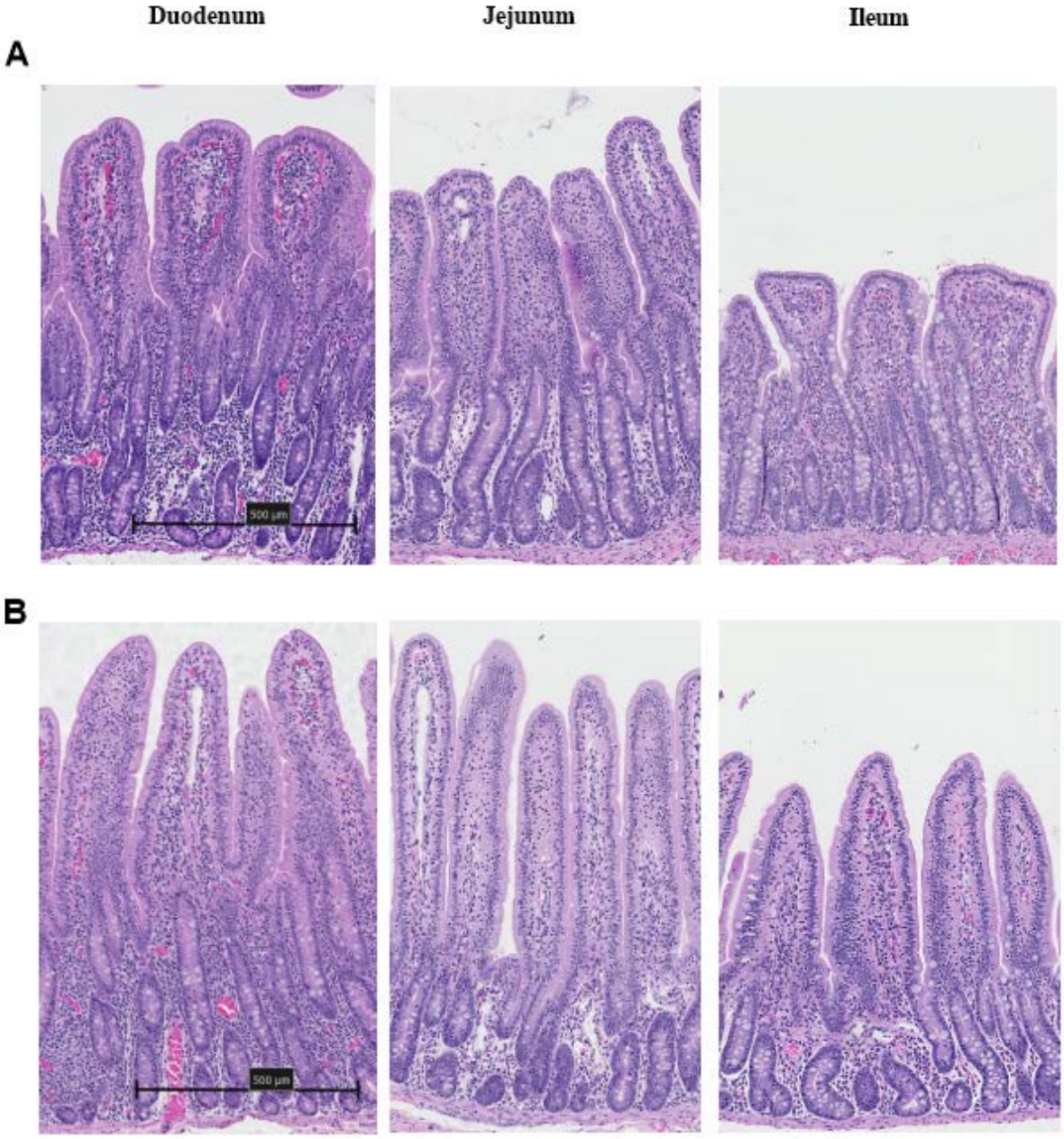


Figure 3.2. Effect of MAP supplementation on villus height (VH) and crypt depth (CD). Representative sections of duodenum, jejunum, and ileum for control piglets **(A)** and MAP-fed piglets **(B)**. Scale bar = 500 µm.

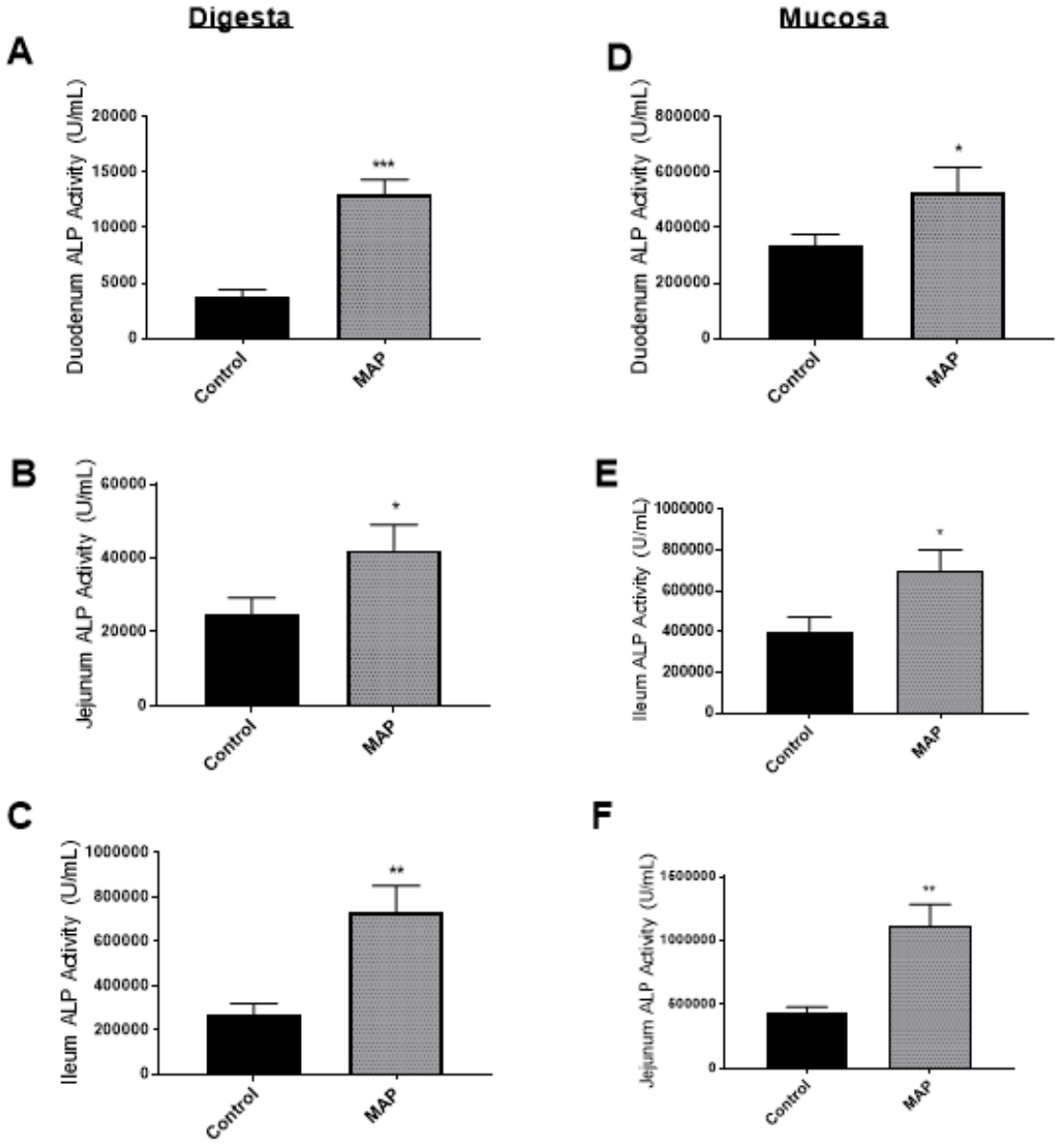


Figure 3.3. Feeding MAP increases alkaline phosphatase activity in the small intestine. MAP-supplemented piglets had increased alkaline phosphatase activity in duodenual, jejnual, and ileal digesta **(A-C).** Mucosal alkaline phosphatase activity in the duodenum, jejunum, and ileum was also increased in weaned piglets supplemented with MAP **(D-F).** Data are represented as mean ± SEM. (n = 8). (*, P<0.05; **, P<0.01, ***, P<0.001)

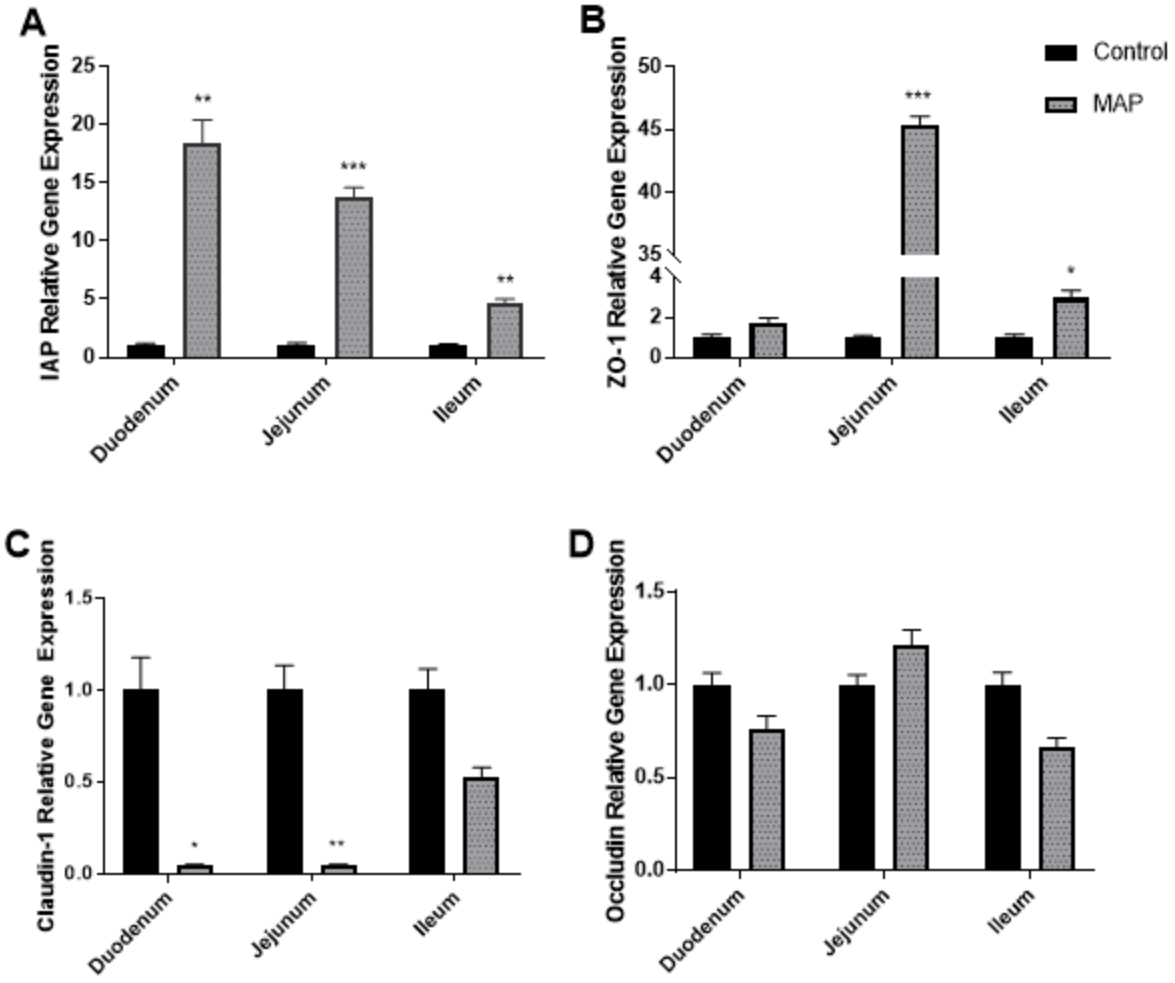


Figure 3.4. Intestinal alkaline phosphatase and intestinal tight junction protein gene expression is protected against weaning-induced downregulation by exogenous MAP. Piglets fed 4,000 IU/kg feed MAP had increased IAP and ZO-1 gene expression as compared to weaned piglets without dietary intervention **(A-B).** Claudin-1 gene expression was decreased in MAP-supplemented piglets **(C).** Exogenous MAP supplementation has no effect on occludin gene expression **(D).** Data are presented as ± SEM. (n = 5-8).
(*, P<0.05; **, P<0.01; ***, P<0.001).

3.5 Discussion and Future Directions

Previous work in our lab has shown that MAP can detoxify LPS from members of Gram-negative bacteria and reduce the inflammatory effects *in vitro* and *in vivo*. In the present study, we found that supplementing post-weaning pigs with MAP protects against weaning-induced growth depression, villous atrophy and crypt hyperplasia, decreased IAP activity and gene expression, and disruption of tight junction proteins.

At weaning, piglets are moved from a highly digestible liquid diet to a less palatable dry diet, and as a consequence, feed intake is reduced, and the piglet has reduced nutrient absorption and utilization leading to reduced growth performance. It is essential to get pigs growing as soon as possible after weaning, and Tokach *et al*. (1992) reported that weight gain in the first few weeks after weaning impacts the total days to market[112]. We found that weaned piglets fed a standard phase 2 diet supplemented with 4,000 IU/kg body weight MAP gained significantly more body weight during the second week of study compared to piglets fed a standard control phase 2 diet. Furthermore, during the second week on the experimental diet, piglets supplemented with MAP had a significantly higher average daily gain. The extent to which this increase in body weight and ADG continue throughout the feeder period or the effects of supplementing MAP immediately post-weaning requires more research. Taken together, these results indicate a novel therapeutic approach to decreasing growth depression in weaning piglets.

Intestinal morphology, including villus height, crypt depth, and VH:CD of the duodenum, jejunum, and ileum can be indicative of overall piglet gut health. Post-weaning, villus height is reduced, and crypt depth increases. Longer villus height has been associated with active cell mitosis and greater nutrient absorption, and the villus height/crypt depth ratio correlates with epithelial turnover[113,114]. Villus crypts provide new villi, and deeper crypts increase tissue

turnover to allow the renewal of villi in response to normal sloughing, inflammation from pathogens, or high demand in a growing animal. In the present study, dietary supplementation of MAP reported an increase in villus height in the duodenum and jejunum and a decrease in crypt depth in the duodenum, jejunum, and ileum. Furthermore, there was a significant increase in the villus height to crypt depth ratio for all segments of the small intestine. To our knowledge, this is the first study of its kind to evaluate the effects of exogenous AP supplementation on gut morphology of post-weaning piglets. Reduced intestinal inflammation from MAP detoxifying bacterial components in the lumen and increased weight gain from enhanced utilization of enteral nutrients may be responsible for the optimal villus height and crypt depth that is seen in MAP-supplemented piglets.

Post-weaning syndrome is also associated with increased mucosal attachment of pathogenic *Escherichia coli* and other Gram-negative bacteria that potentiate the syndrome due to the piglet's immature immune system. Endotoxins, such as Gram-negative bacteria cell wall component lipopolysaccharide (LPS), spontaneously release and can cause structural changes in the small intestine by disrupting the structure of tight junctions allowing pathogens to cross into systemic circulation[61,64]. Under homeostatic conditions, IAP suppresses LPS-mediated inflammatory responses through modulating commensal bacteria and gut pH, detoxifying LPS, protecting gut barrier function, and balancing fluid and electrolyte secretion. It is well known that IAP expression is decreased in weaning piglets and that IAP expression is dependent upon enteral nutrition[75,115]. Therefore, we sought to determine the effects of exogenous AP supplementation on IAP activity in weaning piglets.

Compared to control phase 2 diet fed piglets, MAP-supplemented piglets had significantly higher AP activity in the digesta and mucosa of the duodenum, jejunum, and ileum.

Furthermore, MAP-supplemented piglets had significantly higher IAP gene expression in the duodenum, jejunum, and ileum. Weaning-induced inflammation is marked by upregulation of pro-inflammatory mediators such as TNFα and IL-6, which have been shown to inhibit IAP expression[116,117]. Since weaning is known to increase endotoxin load and LPS is known to upregulate TNFα and IL-6, and one of IAP's biological roles is to mitigate the inflammatory effects of LPS and protect against bacterial translocation; it is possible the increase in IAP activity and expression is due to exogenous MAP detoxifying LPS in the lumen and protecting against inflammatory mediators. AP also create an alkaline microenvironment that is unfavorable for pathogen attachment, and exogenous MAP could potentiate this alkaline environment for optimal commensal bacterial growth which would reduce the concentration of unfavorable pathogens[118,119]. A commensal bacterial environment would protect against inflammation and downregulation of IAP activity and expression.

IAP is a marker for enterocyte differentiation, and weaning is associated with decreased feed intake, which decreases enterocyte differentiation. Since MAP-supplemented piglets had significantly higher average daily gain, the increase in IAP gene expression could be in part due to increased feed intake. Increased feed intake would increase rapamycin-dependent signaling pathways, which would stimulate the global synthesis of intracellular proteins, including IAP. Therefore, MAP-supplementation could increase IAP expression and/or activity in the digesta and mucosa through multiple mechanisms and exerts a protective role against weaning-induced decreases in IAP activity.

In the weaned piglet, downregulation of the IAP gene is associated with poor intestinal integrity[120]. Intestinal permeability has long been considered to be a measure of intestinal barrier function. The intestinal barrier is regulated by a complex system of transmembrane and cytosolic

proteins called tight junction proteins. The most critical components of a tight junction are transmembrane proteins occludin and the claudin family, specifically claudin-1, and the linker protein ZO-1. Occludin is an integral membrane protein having functional roles in maintaining the integrity of the tight junction[121]. Claudin-1 has diverse functions depending on cell type and host but is localized to ZO-1 expression. ZO-1 is a vital intracellular tight junction protein that is essential for tight junction assembly and link the cell cytoskeleton to transmembrane tight junction proteins occludin and claudins[122]. Inflammatory mediators, such as pro-inflammatory cytokine TNFα, are released following LPS-stimulation and increase intestinal permeability by disrupting expression of claudins and occludins and by altering the lipid environment of phospholipid membranes to displace tight junction proteins[65,66] Furthermore, TNFα is thought to mediate effects on tight junctions by downregulating ZO-1 stability and concentration at the junctional surface[123]. In human and mice, exogenous IAP has been shown to prevent the development of colitis, a disease marked by poor intestinal permeability[111,124,125]. Furthermore, IAP treatment has been shown to prevent increased intestinal permeability in a mouse starvation model[126].

In the present study, we found that MAP supplementation significantly upregulated gene expression of tight junction protein ZO-1 in the jejunum and ileum, decreased claudin-1 in the duodenum and jejunum, and exerted no effect on occludin gene expression. Our results agree with Liu *et al*., (2016) which showed that IAP treatment preserved localization of ZO-1 and occludin proteins during inflammation. The same study also showed that IAP gene deletion in mouse embryonic fibroblasts resulted in lower levels of ZO-1, ZO-2, and occludin compared to wild-type controls[127]. Zinc is an important cofactor for optimal AP activity and Zhang *et al*., (2019) showed that zinc supplementation alone was sufficient to enhance ZO-1 expression in

weaning piglets, and zinc-supplemented piglets had a significantly higher ADG[128]. Taken together, these results argue that AP exerts a protective effect on ZO-1 disruption during weaning-induced anorexia and inflammation. Studies have shown a strong correlation between a decrease in ZO-1 and occludin and an increase in intestinal permeability and a decrease in trans-epithelial resistance[129]. Furthermore, recent literature has shown that tight junction protein claudin-1 is increased during intestinal inflammation[130]. In the present study, we found a decrease in claudin-1 in MAP fed piglets, and this may be due to decreased intestinal inflammation as compared to control fed weaned piglets. We saw no change in occludin gene expression, which is in contrast to Liu *et al.*, (2016) which showed IAP preserved occludin expression in mouse models where colitis decreased the expression. These differences could be due to differences in species and the pathology of weaning versus colitis.

Environmental and physiological stressors during weaning lead to a decrease in feed intake and increased intestinal inflammation, which causes changes to intestinal permeability and enzymatic activity. Together, these consequences potentiate each other and drive inflammation and bacterial translocation, which can lead to growth depression and a high mortality rate during piglet weaning. Exogenous MAP supplemented during the post-weaning phase protected piglets by detoxifying LPS in the gut lumen and creating an alkaline environment unfavorable for pathogen attachment and growth. Decreased inflammation during weaning leads to increased nutrient absorption, which protects against further GIT morphological changes and anorexia-induced decreases in tight junction proteins and endogenous IAP. Furthermore, lower concentrations of endotoxins, and subsequently inflammatory mediators, due to exogenous MAP detoxifying LPS prevent further decreases in endogenous IAP and ZO-1, and increases in claudin-1. Maintaining healthy levels of TJP and IAP allows for an increased growth rate of the

weaned piglet, a more robust immune system, and a protected GIT against opportunistic pathogens. Taken together, the results of these studies indicate a novel therapeutic approach to overcoming post-wean growth plateaus, enhancing small intestinal VH:CD ratios, protecting TJP expression in the small intestine during inflammation, and maintaining high levels of IAP to exert immuno-protective effects in the gastrointestinal tract.

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