EFFECT OF LACTIC ACID BACTERIA (PROBIOTICS) ON LOCAL AND SYSTEMIC IMMUNE RESPONSES IN CATTLE

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Chapter I

Introduction

In normal healthy animals, the gastrointestinal tracts are colonized by a complex microflora containing many different species. A balance of these microorganisms in the gastrointestinal tract is important not only in promoting efficient digestion and maximum absorption of nutrients, but also in increasing the capacity of the host in excluding infectious microorganisms and hence preventing disease (Walter *et al.,* 2003). *Lactobacilli* are common inhabitants of animal gastrointestinal tracts and many investigators have reported them to provide various positive health attributes. This group of "good" bacteria is generally called probiotics. Therefore, probiotics can be defined as living microorganisms that upon ingestion in certain numbers exert health effects beyond inherent basic nutrition (Gurner *et al*., 1998). Naidu *et al.,* 1999 describes probiotics as microbial dietary adjuvants that beneficially affect the hosts' physiology by modulating their mucosal and systemic immunity as well as improving the nutritional and microbial balance in their intestinal tracts. Probiotics are potentially useful in the management and treatment of various gastrointestinal diseases including diarrhea, inflammatory bowel disease, and colon cancer (Rolfe, 2000). Enhancement of nonimmunological gut defense barrier by probiotics may include maintenance of normal levels of intestinal permeability and micro-ecology, which is commonly shifted in the event of intestinal infection by pathogenic bacteria.

Vertebrate immune system can mount both innate and adaptive immune response in the event of infection by pathogenic microorganisms. Many types of immune cells are recruited to elicit an immune response and subsequently neutralize the pathogens. These cells include epithelial cells, natural killer cells, macrophages, neutrophils, dendritic cells and lymphocytes. These cells are quickly activated in the event of infection leading to production of an array of humoral mediators. Some may change their physiology and become phagocytic, yet others get involved in antibody synthesis and secretion. These activated immune cells may provide immediate protection against pathogens or promote specific immune responses. Therefore, these cells are useful in the probiotic enhancement of immunologic barrier in the animal gastrointestinal tract.

Probiotics have profound effects on potentiating both arms of immune responses. For instance, oral administration of the probiotic, *Bifidobacterium breve* was shown in mice that had been previously challenged with cholera toxin to promote humoral immunity by enhancing the secretion of immunoglobulin A (IgA) (Yasui *et al.,* 1999). In an investigation conducted by De Simone *et al.,* (1993), bacterial cell wall products were demonstrated to be able to not only enhance the proliferation of immune cells but also induce the expression of proinflammatory cytokines, which are necessary for the maintenance of a stable Th1/Th2 balance. This delicate balance is important for the host immune function as it dictates whether a humoral (antibody production) or a cell-mediated (cytotoxic T-cell) response should be mounted (De Simone *et al.,* 1993). Therefore, the influence of a probiotic strain of bacteria on the mammalian immune system can be easily evaluated through *in vitro* and *in vivo* measurement of cytokines, immunoglobulin production and lymphocyte proliferation.

The use of antibiotics is associated with the emergence of antibiotic-resistant bacteria, which have become difficult to control and have exerted adverse effects on the consumers of animal products. With the above positive attributes of probiotic bacteria on the prevention and disease management, probiotics hold great potential as a better alternative to antibiotics in farm animals.

In this present study, the use of lactic acid-producing bacteria, *Lactobacillus acidophilus,* as a feed supplement in the diet of healthy Holstein calves and its ability to modulate their innate and adaptive immune responses was investigated.

Chapter II

Literature Review

Probiotics

A "probiotic" by the generally accepted definition, is "a live microbial feed supplement, which beneficially affects the host animal by improving its intestinal microbial balance" (Fuller, 1989). This definition was broadened by Havenaar and Huis in't Veld (1992) to a "mono or mixed culture of live microorganisms which benefit man and animals by improving the properties of their indigenous microflora". Although the definition of probiotics is constantly evolving, they are living microorganisms that confer health benefits to their hosts by improving the indigenous microflora (Sanders, 1998; Tannock, 1999). A European Commission concerted action program redefined probiotics as "a live microbial food ingredient that is beneficial to health" (Salminen *et al.,* 1998). In a broadened definition, Naidu et al., (1999) describes probiotics as microbial dietary adjuncts that beneficially affect the host physiology by modulating the mucosal and systemic immunity as well as improving nutritional and microbial balance in the intestinal tract.

Microflora in animals is extremely important in protecting them against pathogenic infections. This fact is evident in the way germ-free animals become susceptible to infections in their intestines. While it takes 10^5 spores of *Clostridium botulinum* to kill mice with functional microflora, only 50 spores are required to kill germ-free mice (Wells *et al.*, 1982).

In vitro, dozens of microorganisms have been shown to have desirable probiotic qualities. However, some ingested bacteria are normally killed in the host stomachs by gastric juices. A small number of strains have been shown to colonize the human gastrointestinal tract in clinical trial (Crabbe *et al*., 1968). Some scholars believe this colonization is a prerequisite for any health benefits to be conferred. The effect of the probiotics on the intestinal ecosystem, may impact the consumer in some beneficial way.

For a given microorganism to function as an effective probiotic, it must have some important properties as in the case of some species of lactobacilli (Reid *et al.*, 1990). The organism should be able to: adhere persistently to intestinal epithelial cells and mucus; reduce and exclude pathogenic adherence to healthy cells; reproduce in such a manner to allow rapid multiplication and colonization; produce reactive agents such as acids, hydrogen peroxides and bacteriocins that can hamper pathogen reproduction and multiplication; be safe, noncarcinogenic and nonpathogenic; resist various microbicides; and form a balanced flora (Reid *et al.*, 1990).

A number of potential benefits arising from changes to the intestinal milieu through the consumption of probiotics have been proposed, including: a) Increased resistance to intestinal infections, by pathogen interference, exclusion, and antagonism; b) reduction in blood pressure; c) reduction in serum cholesterol concentration; d) maintenance of mucosal integrity; e) alleviation of symptoms of lactose intolerance; f) reduction in allergy g) stimulation of phagocytosis by peripheral blood leucocytes; h) immune system stimulation and modulation in human and animals; i) vaginal and urinary tract health; j) modulation of cytokine gene expression; k) adjuvant effect; l) regression of tumors; m) and reduction in carcinogens or co-carcinogen production

Some probiotics produce metabolic by-products that are antagonistic to establishment of pathogenic microorganisms. Strains of *Lactobacillus* may produce lactic acid and hydrogen peroxide that are not only toxic but might aid in inhibition and exclusion of potential pathogens (Gilliland and Speck, 1977). Perhaps, surprisingly, despite this impressive list of potentially beneficial attributes, probiotics are not commonly part of the medical practitioners' and veterinarians' armamentarium treatment or preventative methods.

The gut mucosal barrier

The small intestine is constantly exposed not only to food but also to a variety of antigens in the life of mammals. These exposures pose challenges to the host because some of the antigens may be pathogenic. The bacterial load in the small intestine is dynamic and keeps changing both in numbers and types. A well functioning mucosal barrier in the gut is important and may help in excluding establishment of pathogenic microoragnisms (Janeway *et al.,* 1999)**.** Some antigens are absorbed across the mucosal epithelial-cell layer and may be processed in the lysosomes or may be eliminated by the mucosal immune system (Isolauri *et al.,* 1993).

It is well documented that the effect of commensal probiotic bacteria on the immune response of their host animals is through their close association with the gut lymphoid tissues found in the intestinal mucosa. The gut-associated lymphoid tissues are found in specific compartments in the host intestinal tract. The Peyer's Patches, which are areas rich in B-cell lymphoid follicles, are highly organized lymphoid tissues in the wall of the small intestine. The Peyer's Patches are also resident to interfollicular populations of T-

cells. Large numbers of lymphoid and myeloid cells aggregate to form the lamina propria, which is found to be rich in immunoglobulin-A plasmablasts. The lamina propria is found scattered under the gut epithelium. Interspersed within the enterocyte monolayer are intraepithelial lymphocytes, which are made up of mostly $CD8^+$ T-cell subsets (Janeway *et al.*, 1999). It has been reported by various groups that the quantity and quality of immune cells in the Peyer's Patches and lamina propria compartments depend on continuous stimulation provided by endogenous intestinal microflora (Cebra, 1999).

In an investigation by Crabbe *et al*. (1968), they demonstrated that colonization of germ-free mice with different strains of lactic acid bacteria (LAB) induced the secretion of mucosal IgA from both the Peyer's Patches and lamina propria. Since these LAB are also commensal organisms in many animals, they might be able to potentiate various indices of hosts' humoral immune responses when used as dietary supplements.

The development of probiotics for farm animals is based on the knowledge that gut microflora is involved in resistance to disease in mammals (Gill, 1998). In a clinical study with human volunteers, Alander *et al.* (1999) demonstrated that *L. rhamnosus* GG attached to human intestinal mucosa and the attachment persisted for about seven days following withdrawal of the probiotics. In a different clinical study, Miller *et al.*, (1993) demonstrated that application of the same strain to premature infants did not decrease pathogen load even if it colonized the intestines. This investigation suggested that the probiotic was poorly antagonistic to pathogenic bacteria *in vivo*. However, the increased likelihood of gastrointestinal infection in infants may be due to immaturity of their gut defense barrier. This barrier is important because it forms a protective phase between the

infants' internal environment and the potentially pathogenic factors in the external environment (Juntunen *et al.*, 2001).

Although the mechanisms behind the positive effects of probiotics on the host are not well known, one way in which probiotics may augment favorable health outcome on the host is by enhancing nonspecific (innate) and antigen-specific (adaptive) immune response (Miller *et al.*, 1993; Juntunen *et al.*, 2001).

Immune modulation by probiotics

Immune modulation by dietary bacteria has continued to be a subject of growing interest. Probiotics have been reported to facilitate stabilization of gut microflora and hence enhance gut defense against pathogenic microorganisms in a way that is nonimmunological (Salminen *et al.*, 1998). They can also enhance immunologic barrier of the host intestine (Kaila *et al.*, 1992). Although it is known that immune function tends to decline with age, supplementation twice daily with a probiotic *Bifidobacterium lactis* was found to significantly increase and improve various indices of immune function in a group of healthy elderly people in a double-blinded trial. The good attributes of the bacterial supplementation were observed in this group after about six week of trial time (Aruchalam *et al.*, 2000). In other studies, probiotics have been shown to augment the humoral arm of adaptive immunity and subsequently enhance the immunologic barrier of the intestine (Kaila *et al.*, 1992).

In the event of their action in animals as immune modulators, probiotics may either have up- or down-regulatory effects on various indices of immune response. Live probiotic microorganisms may stimulate production of cytokines and therefore enhance

natural immune responses (Marin *et al*., 1997). Intestinal inflammation can be suppressed by viable probiotics after oral administration, which makes these microorganisms useful in controlling hypersensitivity reactions (Majamaa *et al*., 1996).

In another independent investigation on the antiproliferative effects of five strains of probiotic bacteria, Pessi *et al.* (1999) studied the proliferation activity of mononuclear cells induced by phytohemagglutin as a mitogen. Proliferation of these cells was carried out in the presence or absence of either unheated or heat-treated probiotic homogenates from *L. rhamnosus* GG, *B. lactis*, or *L. acidophilus*. They demonstrated that homogenates from these bacteria suppressed proliferation of mononuclear cells with mitogen treatment. The suppression was observed with both unheated and heat-treated homogenates. Their findings suggested that these homogenates might be used to generate nonviable food products that are immunologically active.

Low cost and convenience of administration makes immune modulation via the oral route a very desirable treatment. Due to large volumes of antigen required for oral immunization, and that exposure to soluble protein antigen may induce oral tolerance, researchers are leaning towards the use of microorganisms to induce the desired immunity (Wells *et al.*, 1996). The adherence to intestinal epithelium and mucus by probiotics may be an integral factor in the stimulation of the host immune system. Adhesion assists the bacteria in surviving host secretions and improves remarkably the chances of the probiotics colonizing the intestinal mucosa (Sami *et al.*, 2001). When newborn ruminants were inoculated with a combination of pure probiotic cultures, colonization of the gut tissues was observed, and consequently the animals were better

endowed to resist infection by enterotoxigenic *Escherichia coli* (Cheng and Costerton, 1988).

Colonization is very important for the creation of balance of the intestinal microflora. Since bacteria adhere to mucus as well as to epithelial cells, it is believed that mucus has receptors mimicking the epithelial cells. Therefore, mucus can inhibit bacterial adhesion to epithelium. Although mucus serves as a protection to the intestinal mucosa from pathogenic microorganism infection, it also provides binding sites as well as nutrients for the bacteria. These factors may allow bacteria to multiply, which is a positive attribute towards probiotic colonization. Since mucus is continuously sloughed off into the intestinal lumen, and is replaced with new mucus secreted by goblet cells, bacteria inhabiting the mucus layer can only establish large populations when their multiplication exceeds the turnover and loss of the old mucus (Beachy, 1981).

In a study of adhesion of *Enterococcus faecum* 18C23 to porcine intestinal mucus and its ability to inhibit adhesion of pathogenic *Escherichia coli* K88, Jin *et al*. (2000) reported that 90% of inhibition was achieved when 10^9 CFU/mL of *E. faecum* (18C23) culture was added at the same time with *E. coli* K88 to immobilized mucus. Such inhibition of adhesion of *E. coli* K88 by *E. faecum* (18C23) might occur through steric hindrance.

Since adhesion to intestinal mucosa is paramount to establishment and colonization of host intestines by probiotics, Ouwehand *et al*., (2003) investigated the influence of disease on the capacity of bacterial mucosal adhesion. Using six strains of lactic acid bacteria, they observed that all strains adhered better to immobilized mucus than to whole intestinal tissue. However, *L. rhamnosus* and *L. reuteri* were found to show disease-

specific adhesion to intestinal tissue. All strains, except *L. rhamnosus*, displayed disease-specific adhesion to intestinal mucus. Their results strongly suggested that the strains, which bind better for a particular intestinal disease, could be selected for use as probiotics.

Asahara *et al.*, (2001) investigated the antimicrobial activity of intraurethrally administered *L. casei* in a murine model of *E. coli* urinary tract infection (UTI). They observed that a single administration of *L. casei* Shirota at 10^8 CFU 24 hours before a three-week challenge with *E. coli* at 10^6 CFU in the urinary tract dramatically inhibited pathogen growth and inflammatory responses in the urinary tract, suggesting that *L. casei* Shirota may be used as a prophylactic agent for UTI.

The role of probiotics in innate immunity

In mediating the innate or non-specific immunity in host animals, several types of immune cells are recruited and stimulated. These cells include neutrophils, eosinophils, macrophages, epithelial cells, natural killer cells and M cells. These cells monitor sites of pathogen entry and mount nonspecific immune responses including phagocytosis and natural killing (Janeway *et al.*, 1999). It is reported that innate immune responses in animals can be modulated by consumption of specific lactic acid bacteria (Perdigon *et al.*, 1995; Schiffrin *et al.*, 1995). In a study conducted by Haller *et al.* (2000), human peripheral blood mononuclear cells (PBMCs) and purified lymphocyte subsets were capable of being stimulated by non-pathogenic gram-positive species of *Lactobacillus*. *In vivo*, interaction between nonpathogenic commensal bacteria and blood leukocytes may occur in definite compartments of the host's mucosal immune system. They also reported

that increase in the number and immune function of lymphoid effector cells at the mucosal level is heavily dependent on the presence of gut microflora. Secretion of interferon-γ (INF-γ) from purified natural killer (NK) cells was significantly increased in the presence of macrophages primed with bacteria, suggesting that probiotic bacteria are important in the activation of NK cells by macrophages (Haller *et al.*, 2000).

It is well documented that there is recirculation of the mucosal immune system. Cells of the mucosal-associated lymphoid tissue (MALT) recirculate within the mucosal system. Given that there is evidence of translocation of nonpathogenic bacteria through the epithelial barrier via M-cell pockets, there is every chance that these bacteria interact with several types of immune cells, which are resident in M-cell pockets. Probiotic bacteria might exploit this fact to potentiate host immune function (Neutra, 1999).

Phagocytosis is an important arm of the innate immune system and is among the first line of defense that animals employ in the event of infection by pathogenic microorganisms. Phagocytic cells are known to produce agents that are toxic to pathogens like reactive oxygen intermediates and lytic enzymes. They are involved in engulfing and destroying particulate antigens. Phagocytes may play an integral role in initiating inflammatory reactions. Salminen *et al.* (1998) reported that intestinal inflammation might be as a result of imbalance in the intestinal microflora. Gill *et al.*, (2001) showed that both live and heat-killed preparations of *L. rhamnosus* HN001 had the capacity to enhance the phagocytic activity of blood and peritoneal leukocytes in mice after administration of 10^9 CFU of the microorganism daily. They observed that the enhancement of the phagocytic capacity was in a dose-dependent manner. However, they also demonstrated that mucosal antibody responses were enhanced by live but not killed

L. rhamnosus HN001. Their results suggest that while innate immunity is responsive to both killed forms of bacteria, only live forms are able to stimulate the gut mucosal immunity. In an earlier study, Gill *et al.* (2000) demonstrated that feeding mice *L. acidophilus* or *B. lactis* resulted in a significant increase in the phagocytic activities of both PBMCs and peritoneal macrophages compared with control mice.

An in vitro investigation conducted by Pinchuk *et al.* (2001) demonstrated that a probiotic strain, *Bacillus subtilis* 3, was able to secret antibiotics that inhibited growth of *Helicobacter pylori*. They showed that the anti-*H. pylori* activity in cell-free supernatants was heat stable and protease insensitive. Their result suggested that other probiotic preparations might exhibit similar activities against pathogenic microorganisms.

The role of probiotics in acquired immunity

Acquired or adaptive immunity is the response of antigen-specific lymphocytes to antigen and includes the development of immunological memory. Adaptive immune responses are generated by clonal selection of lymphocytes (Janeway *et al.,* 1999). Acquired immunity involves lymphocytes with receptors for specific antigens and presentation of the antigens in the context of two different major histocompatibility complexes (MHC) by antigen presenting cells (APCs). As a result, subsets of helper T-cells (Th), the main effectors and regulators of cell-mediated immunity may be activated (Roitt *et al.,* 1985). Upon activation by antigen or pathogen, T-cells are activated to synthesize and secrete a variety of cytokines that serve as growth, differentiation and activation factors for other immunocompetent cells. The types of cytokines produced

during infection are key factors in determining whether a humoral (antibody production) or a cellular immune response is potentiated in the host animal.

Among the first proinflammatory cytokines expressed by host immune cells after pathogenic challenge are tumor necrosis factor-alpha (TNF-α), interleukin 1β (IL-1β), IL-6 and interferons (IFNs) (Tracey and Cerami, 1993). It is well documented that cytokines produced later during microbial infection will either influence the development of cell-mediated immune response associated with T-helper type 1 cells (Th1) or a humoral immune response that is associated with T-helper type 2 cells (Th2) (Abbas *et al.*, 1996; Romagnani, 1996).

Among major Th1 type cytokines are IL-12, IFN-γ and IL-18 of which IL-12 is known to stimulate IFN-γ production in both T and NK cells. It also promotes the development of naïve $CD4^+$ T cells into Th1 type cells (Abbas *et al.*, 1996; Trinchieri, 1995). Phagocytic cells are known to enhance their IL-12 production after stimulation with IFN-γ, which has a down-regulatory effect on Th2 type cell proliferation and activation (Kohno and Kurimoto, 1998). Acting synergistically with IFN-α and IL-12, IL-18 is able to enhance IFN-γ expression in T cells (Sareneva *et al.*, 1998). The development of naïve T cells to active Th2 type can be enhanced by IL-4 (Seder and Paul, 1994). IL-10, which is produced by both macrophages and lymphocytes, can enhance Th2 immune responses by inhibiting the production of TNF-α, IL-1β, IL-6, IFN-γ and IL-12 (Abbas *et al.*, 1996; D'Andrea *et al.*, 1993; De Waal *et al.*, 1991).

Some have shown that LAB are able to induce the production of IFN-γ, IL-6, IL-10 and TNF-α in human lymphocytes (Miettinen *et al.*, 1996). Von der Wied *et al.* (2001)

investigated the capacity of LAB to antagonize specific T-helper cell function *in vitro* and subsequent prevention of inflammatory intestinal immunopathologies. Their results showed that all LAB strains used were able to induce murine splenocytes to produce both IL-12 and IL-10. It is well known that IL-12 and IL-10 have the potential for induction and suppression of Th1 functions, respectively. Among all LAB strains used, *L. paracasei* NCC2461 induced the highest levels of both IL-10 and IL-12. They also showed that the proliferative activity of $CD4^+$ T cells was strongly inhibited in a dose-dependent manner by *L. paracasei*. In a further investigation, they demonstrated that this bacterium inhibited the secretion of Th1 and Th2 effector cytokines by $CD4^+$ T cells, maintained the production of IL-10 and induced the secretion of transforming growth factor-β (TGF-β) by $CD4^+$ T cells (Von Der Weid *et al.*, 2001).

In a study conducted by Pavan *et al*. (2003), they used a mouse model to evaluate the persistence, safety and immune modulation capacities of LAB. They demonstrated that TNF-α, IFN-γ, IL-10 and IL-4 were induced in healthy mice that received a single dose of *L. plantarum* NCIMB8826 or *L. lactis* for four consecutive days. When mRNA transcripts were assessed for these cytokines by semi-quantitative reverse transcriptase PCR, they found that there was higher expression of IFN-γ in the colon than in the ileum. However, the levels of IL-4 and IL-10 expression were significantly higher in the ileum than in the colon. Taken all together, these results suggested that there was a difference in Th1/Th2 balance between ileum and colon in mice. Since the bacterium persists in the mouse gut, it might be a suitable probiotic candidate for treatment of chronic inflammation.

Gill *et al.*, (2000) observed that feeding healthy mice with 10^9 CFU of *L. acidophilus*, *L. rhamnosus* or *B. lactis* enhanced the proliferative responses of spleen cells to concanavalin A (Con A) and lipopolysacharide (LPS), which are T-and B-cell mitogens, respectively. The spleen cells from mice given these different bacteria expressed greater amounts of INF-γ after stimulation with Con A than cells from control mice. When they assayed the levels of antibody responses after either oral or systemic administration of antigen, they found the levels to be higher in mice given the bacteria than in control mice. Their results suggested the use of LAB as feed supplements in mice was able to enhance several factors of both humoral and cellular immune responses.

Ibnou-Zekri *et al.*, (2003) investigated the differential impacts of *L. johnsonii* and *L. paracasei* on the development of mucosal and systemic antibody responses in mice. Despite the fact that these two organisms had similar growth and adherence capacities to enterocytes *in vitro*, they showed marked differences in their patterns of colonization and translocation. They also promoted different immune responses at the mucosal and systemic levels *in vivo*. They demonstrated that of the strains tested, *L. johnsonii* colonized the intestines more efficiently than did *L. paracasei* in mice and that both strains activated mucosal B-cell responses evidenced by aggregation of cells of the Peyer's Patches. They also showed that IgA secreting plasma cells were prevalent in lamina propria after association with either of the bacteria strains. Germ-free mice had either few or no IgA secreting cells even after association with either of the bacterial strains. They further showed that mice associated with *L. johnsonii* but not *L. paracasei* secreted increased amounts of *Lactobacillus*-specific IgA.

Waard *et al.* (2001) investigated the effects of orally administered viable *L. casei* Shirota on immune response indices of Wister and Brown Norway rats. They used the *Trichinella spiralis* host resistant model. In their study, two weeks before and after *T. spiralis* infection, rats were fed with 10^9 CFU of *L. casei* 5 days per week. They observed that *T. spiralis*-specific delayed-type hypersensitivity (DTH) responses were significantly augmented in mice fed *L. casei* than in control mice and significantly enhanced *T. spiralis*-specific antibody IgG2b in both types of rats. This type of DTH response is considered to be a manifestation of Th1 cell-mediated immunity.

In an investigation on whether *L. casei* Shirota exhibited any activity against the influenza virus, Hori *et al.* (2001) observed that mice given the bacterium intranasally had greater expression of IL-12, IFN-γ, and TNF-α in their mediastinal lymph node cells. This high expression of cytokine helped in excluding the influenza virus. They observed that viral titers in the nasal washes of mice given the bacterium before infection with the pathogenic virus were significantly lower than in those not inoculated with the bacterium. They also observed that the survival rate of the mice inoculated with the bacterium was higher than that of the mice not inoculated, after both groups were challenged with the virulent virus. They concluded that because the viral titers were decreased in mice inoculated with *L. casei* Shirota to about a tenth of the viral titers found in the control group, administration of the bacterium enhanced cellular immune response in upper respiratory tract of mice and conferred protection to them against the influenza virus infection (Hori *et al.*, 2001). Yasui *et al.*, (1999) reported that another probiotic bacterium, *B. breve* YIT 4064, was able to potentiate humoral immune response and that oral administration of this strain was able to augment production of antigen-specific

immunoglobulin G in serum and conferred protection against influenza virus infection in mice.

Matsuguchi *et al.*, (2003) investigated the stimulatory effects of *Lactobacillus* species on mouse immune cells. In their investigation, six heat-killed cultures were used, including *L. fermentum, L. rhamnosus, L. casei, L. plantarum, L. acidophilus* and *L. reuteri*. They observed that splenic mononuclear cells from BALB/c mice were induced by all six strains to produce TNF-α in varying amounts. They also observed that the protoplast fractions of *Lactobacillus* had the highest activity for TNF-α induction and NF-κB activation in a macrophage cell line RAW264.7. In a further experiment they observed that Toll-like receptor-2 (TLR2) but not TLR4 was essential for induction of TNF-α by *Lactobacillus* lipoteichoic acid (LTA). They also demonstrated that TLR2 mediated the activation of NF-κB by *Lactobacillus* LTA and that TLR2 was essential for cell response to *Lactobacillus* strains but not TLR4 including eliciting of proinflammatory reactions.

Selection of probiotic strains

Some of the most important factors to consider when selecting a probiotic strain are to screen their capabilities for mucosal adhesion, mucosal penetration, inhibition of pathogen growth, resistance to bile, resistance to peristaltic gut movements, and tolerance to low pH (Sami *et al.*,2001). It is established that interaction with mucus is among the initial steps in adhesion of bacteria to the intestinal mucosa and other surfaces. This interaction may promote competitive exclusion by probiotic microorganisms by competition for nutrients, blocking adhesion receptors and production of antimicrobial

substances**.** The overall result is blockage of entry sites for host pathogens (Sami *et al.*, 2001). The choice probiotic, therefore, should be one that is able to multiply fast and out-compete some of the hosts' resident microflora.

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Chapter III

Modulation of Immune Responses in calves by Lactic Acid Bacteria (Probiotics)

Abstract

Some probiotic bacteria can favorably alter the balance of intestinal microfloral, inhibit the growth of pathogenic bacteria, promote nutrient digestion, increase resistance to infection and boost immune function. Two experiments were conducted to investigate the immune modulatory effects of feeding 3 to 4 month-old healthy Holstein female calves with 10^9 colony-forming units (CFU).calf^{-1}.d^{-1} of *Lactobacillus acidophilus* 381-IL-28. In the first experiment, a total of eight animals were divided into two groups with four calves having their diets supplemented with the bacteria, while the other four served as controls. On days 0, 2, 4, 7, 14, and 21, animal weights were recorded, and fecal samples and peripheral venous blood were collected from each calf. In the second experiment, six calves were supplemented with probiotics in their diets, while the other five served as controls. On days 0, 7, 14, 21, 28, and 35, periperal venous blood was obtained from the animals. Fecal temperatures and fecal pH were recorded and animals were weighed. In both experiments, differential white blood cell counts were determined. White blood cells were isolated and their phagocytosis capacity was determined by flow cytometry. Total RNA was also extracted from white blood cells, and used to measure the cytokine gene expression by semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). In addition, serum IgA levels were measured by enzyme-linked immunosorbent assay (ELISA). The results indicated that there was no statistical difference between probiotic treated and control animals in weight gain, fecal

temperature, fecal pH, phagocytosis capacity, differential white blood cell counts, and cytokine expression levels in white blood cells throughout different sampling times in either experiment. There also were no significant difference between serum IgA of the two groups of animals in either experiment. Taken together, these results suggested that feeding healthy Holstein calves with 10^9 CFU.calf^{-1}.d^{-1} of *L. acidophilus* was not sufficient to augment either innate or adaptive immune responses. A different dose or strain of probiotics may be needed in the future to evaluate their effect on immunity of Holstein calves.

Introduction

For the host to be protected against infection, it must have a normal intestinal microflora, which serves as an extremely important barrier against pathogenic microorganisms (Wells *et al*., 1982). Oral consumption of health-promoting lactic acid producing bacteria or probiotics has been associated with prevention, alleviation or cure of diverse intestinal disorders (Alander *et al*., 1999). It is generally accepted that use of a probiotic, defined by Fuller as "a live microbial feed supplement which beneficially affects the host animal by improving its microbial balance" (Fuller, 1989), is an interesting approach for prevention and treatment of some infectious intestinal diseases (Banasaz *et al.*, 2002). In recent years, data based on clinical studies indicating health-promoting properties of several probiotic strains have started to accumulate (Lee and Salminen, 1995). Although many mechanisms have been proposed by which probiotics promote intestinal health and overall well-being of the host animal, one of the most important mechanisms is modulating host immune function (Isolauri *et al*., 2001).

The mammalian immune system is capable of mounting both the innate and adaptive immune responses when challenged by a pathogenic microorganism. The gastrointestinal tract of mammals is resident to various immune cells including epithelial cells, macrophages, lymphocytes, neutrophils, natural killer cells and dendritic cells. All these cells are aggregated in Peyer's Patches, lamina propria and intraepithelial regions (Janeway, 1999). These immune cells are quickly activated upon infection leading to enhanced phagocytosis and production of various humoral mediators, which collectively provide immediate protection for the host or initiate the development of specific immune responses (Zhang and Ghosh, 2001). Some of the humoral mediators produced upon infection include cytokines, which will promote a Th1 or a Th2 immune response (Infante-Duarte and Kamradt, 1999). Probiotic bacteria have been shown to affect innate, humoral and cellular arms of the immune system. Oral administration of Lactobacilli resulted in enhanced phagocytosis and natural killer activities as well as elevated production of IgA and decreased IgE production in both humans and animals (Isolauri *et al*., 2001).

Gastrointestinal diseases continue to cause significant economic losses in the bovine industry. Diarrhea is one of the most common causes of neonatal mortality in cattle. Poor growth performance, which normally accompanies diarrhea and subsequent low feed efficiency, are areas of major concern to the bovine industry. Most of the diarrhea is caused by enteric pathogens such as *Escherichia coli* and *Salmonella* (Levy, 2000). In the recent past, there has been an abated emergence of antibiotic-resistant bacteria, which is not only posing serious concerns to public and health specialists, but also to food animal producers (Teuber, 2001). Consumers have continued to criticize the practice of

using massive antibiotics in animal feeds as growth promoters and means of disease control (Threlfall *et al*., 2000). The unchecked use of antibiotics could increase the prevalence of antibiotic-resistant bacteria in the environment, and there is a fear of contaminating animal food products with drug-resistant pathogens. The use of probiotics in disease management and treatment could serve as an alternative to the use of antibiotics. Evidence continues to accumulate about the beneficial effects of probiotics on the prevention and treatment of gastroenteric diseases (Marteau *et al*., 2001).

In the present study, two animal experiments were conducted to investigate the effects of lactic acid-producing bacteria, *L. acidophilus,* on local and systemic immune responses in cattle. We hypothesized that *L. acidophilus* is capable of influencing both mucosal and systemic immune responses by modulating differential white blood cell counts and phagocytic activity of white blood cells as well as the production of immunoglobulins, cytokines and disease resistance molecules in Holstein calves.

Material and Methods

Lactobacillus acidophilus preparation

The probiotic used in this study was *L. acidophilus* 381-IL-28, which was kindly provided by Culture systems, Inc. (Mishawaka, IN) in a lyophilized form with lactose as a carrier. Each bag contained enough bacteria to feed 4-6 calves with 1×10^9 CFU.calf^{-1}.d^{-1}.

Experimental design

Two independent animal trials were conducted in accordance with and approved by the Oklahoma State Institutional Animal Care and Use Committee. In the first animal trial, eight healthy, 3-month-old, female Holstein calves were (initial BW = 97 ± 22 Kg) divided into two groups based on their weight and ancestry and housed in the Nutrition and Physiology Research Center at Oklahoma State University. The calves were fed twice daily with 4 kg/d of a standard mixed grain ration without antibiotics, but supplemented with rumensin, an ionophore with bacteriostatic effects. The animals were allowed free access to water and alfalfa hay cubes. After two weeks of acclimatization, 1 x 10^9 CFU.calf^{-1}.d^{-1} of *L. acidophilus* 381-IL-28 were added to the mixed grain ration and fed individually to 4 calves. The other 4 calves did not receive any *Lactobacillu*s but were fed the mixed grain with the addition of an amount of lactose comparable to that used as carrier for the dried cells of *L. acidophilus*; these animals served as the control group. The trial was conducted for three weeks. Samples from the animals were collected on d 0, 2, 4, 7, 14, and 21 following acclimatization.

The second trial involved a total of 11 healthy, 3-month old female Holstein calves (initial BW = 185 ± 30 Kg). They were divided into two groups based on their weights and ancestry and housed in the Nutrition and Physiology Research Center. The calves were fed twice daily with 4 kg/day of mixed grain ration without both rumensin and antibiotics. After three weeks of acclimatization, 1 x 10^9 CFU.calf^{-1}.d^{-1} of *L. acidophilus* 381-IL-28 were added to the mixed grain ration and fed individually to six calves. The other five calves did not receive any *Lactobacillu*s but were fed mixed grain with the addition of the same amount of lactose and these animals served as the control group. On

d 0, 7, 14, 21, 28 and 35, the calves were weighed and their weights recorded. Their rectal temperatures, taken with a clinical thermometer, were recorded. At the same time, 10 mL of whole blood and 40 mL of uncoagulated blood were collected from the jugular vein using vacuum tubes. Heparin was used as anticoagulant. These blood collection procedures were done on the calves secured in a chute. The sampling procedures were carried out in the mornings before the animals were fed.

Isolation of white blood cells by hypotonic lysis of red blood cells

White blood cells were isolated from uncoagulated blood by hypotonic lysis as described previously (Zhang *et al.,* 1997; Zhang *et al.,* 1999). Basically, hypotonic lysis of erythrocytes was done by addition of 0.2% sterile NaCl to the blood with gentle mixing for about 30 to 40 seconds followed by addition of an equal amount of 1.6% NaCl. The mixture was centrifuged (500 x g) and cell pellet was saved. Two or three rounds of lysis and centrifugation were carried out until a white pellet of cells was obtained. The cell pellet (i.e., white blood cells) was then resuspended in 4 mL of cell culture medium, RPMI1640, counted with a hemocytometer, and maintained on ice.

Phagocytic activity of the white blood cells

Phagocytosis assays were conducted in accordance with the Vibrant Phagocytosis Assay Kit (V-6694) from Molecular Probes, Inc. (Eugene, OR). In general, 1 mL of isolated white blood cells (10^6 cells/mL) from each calf were mixed with 2 μL of fluorescein-labeled *E. coli* (K-12 strain) and incubated with gentle rocking on a platform (Midwest Scientific, Model: Reliable Scientific, St. Louis, MO) at 37^{o}C for 30 minutes.

After incubation, the cells were centrifuged at 500 x *g* for 5 minutes at 4^{o}C and the cell pellet was washed twice with 2 mL of cold PBS. The cells were then fixed in 1 mL of 2% formaldehyde on ice for 10 minutes. Trypan blue (50 µL of 4 mg/L) was added to each tube and mixed by vortexing and then analyzed by a FACS flow cytometer (Becton Dickson, Model: FACS caliber, Franklin Lakes, NJ). The negative controls were viable cells resuspended in RPMI1640 without the addition of fluorescein-labeled *E. coli*. Each experimental sample was done in triplicate.

Differential white blood cell count

All procedures used during the differential white blood cell counts were conducted in accordance with PROTOCOL^{TM} Hema–Qick III Kit for Wrights-Giemsa staining from Fisher Diagnostics Inc. (Middletown, VA). A drop of blood without anticoagulant was used to make the blood smear. The smear was let to dry at room temperature and later stained with Hema III stain kit. The slides were then observed under a light microscope using the oil immersion objective. The first 100 cells were identified and number of each cell type recorded.

Measurement of gene expression levels by reverse transcriptase-PCR (RT-PCR)

Total RNA was isolated from about 10^7 cells using the Trizol Reagent (Invitrogen, Life Technologies, San Diego, CA) and subsequent RT-PCR was carried out essentially as described previously (Zhang *et al.,* 1997; Zhang *et al.*, 1999). The first-strand cDNA synthesis were conducted with ImProm-II Reverse Transcriptase (Promega, Madison, WI) with 4 µg of total RNA. The PCR was done in a total of 20 µL with 0.5 µL of first-

strand cDNA in the presence of specific primers for either house-keeping gene (β-actin) or genes of interest (Table 1). The PCR was performed by using the following program: 94^{o}C for 2 minutes, and then different cycles of 94^{o}C for 30 seconds, 55^{o}C for 30 seconds, and 72^{o}C for 1 minute.

Table 1. A list of gene-specific primers used for cytokine gene RT-PCR

IL-Iβ Sense primer: GTGTTCTGCATGGAGCTTTGTG Antisense primer: GCTTTCT (/C) TTAGGGAGAGAGG PCR product size: 349 bp
IL-8 Sense primer: CTCTCTTGGCAGC (/T) TTTCCTG Antisense primer: TCTGCACCCACTTTTCCTTGG PCR product size: 237 bp
IL-10 Sense primer: TTACCTGGGTTGCCAAGCCT Antisense primer: TTGTAGACACCCCTCTCTTGG PCR product size: 240 bp
IL-12p40 Sense primer: TCAGGGACATCATCAAACCAG Antisense primer: GACACAGATGCCCATTCACTC PCR product size: 286 bp
IL-18 Sense primer: CCTGGAATCA (/G) GATC (/T) ACTTTGG Antisense primer: TACACTGCACAGAGATGGTTAC PCR product size: 218 bp
IFN-γ Sense primer: AACCAGGC (/T) CATTCAAAGGAGC Antisense primer: GAAATAGTCACAGGATACAGG PCR product size: 437 bp
iNOS Sense primer: ACTTGGCTAACGGAACTGGAC Antisense primer: TTCTGGTGAAGCGTGTCTTG PCR product size: 259 bp
TGF-β Sense primer: CAACTACTGCTTCAGCTCCAC Antisense primer: CGCACGATCATGTTGGACA PCR product size: 309 bp
NRAMP1 Sense primer: ACAGCAGCCTCCACGACTAC Antisense primer: TTCAGGAAGCCCTCCATCACA

PCR product size: 205 bp

ELISA analysis of IgA and levels in serum

The serum samples were prepared from peripheral venous blood collected in 10 mL vacuum tubes without anticoagulant. The blood was let to coagulate at 4 ^{0}C and then centrifuged at 500g. The supernatant (serum) was aspirated and saved at –20 ^{0}C for future IgA assay. ELISA assays of for IgA levels in serum were performed with ELISA Kits from Bethyl Laboratories (Montgomery, TX) according to the manufacturer's instructions. Briefly, 1 µL capture antibody was diluted to 100 µL in the coating buffer (0.05 M Carbonate-Bicarbonate, pH 9.6), added in 96 well microplates, and incubated for 60 minutes at room temperature. After incubation, the excess capture antibody was aspirated from each well and the wells washed with wash solution (50 mM Tris, 0.14 *M* NaCl, 0.05% Tween 20, pH 8.0) three times. The coated plate was then blocked with 200 µL of blocking solution (50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH 8.0) for 30 minutes at room temperature followed by washing three times with wash solution. Bovine serum albumin (BSA) was added to the blocking solution.Serum samples were thawed on ice and diluted to 1:10,000. Standards were diluted in the same sample diluent according to the instruction**.** One hundred microliters of the samples or standards (from the kit) were added to the assigned wells. Standards, samples, and blanks were analyzed in duplicate. The plates were then incubated for 60 minutes at room temperature. After incubation, the samples and standards were removed by aspiration and the wells washed five times with wash buffer.

Horseradish peroxidase (HRP) conjugated capture antibody was diluted in the wash buffer to 1:35,000. To each well, 100 µL of the diluted HRP conjugate was added and

then the plate incubated for 60 minutes. The plates were washed five times with wash solution. The color was developed by adding 100 µL/well of the substrate solution, 3,3" 5,5" tetramethylbenzidine (TMB), followed by incubation at room temperature for 15 minutes, after which the reaction was stopped by the addition of 100 µL of 2 M sulphuric acid. The color was read at 450 nm using a microtiter plate reader (Molecular Devices, Sunnyvale, CA). The averages of the duplicate readings from each standard, control and sample were calculated. The Blank reading was subtracted from each of the averages. A standard curve was then generated for standards. The IgA concentration of each sample was then extrapolated from the standard curve.

Statistical Analyses

Proc Mixed analysis of Statistical Analysis System (SAS Institute, Cary, NC) was used. Experiments 1 and 2 were analyzed separately. Animal served as the experimental unit. Data are presented as Least squares (LS) means ± standard error. All data were analyzed as a completely random design with repeated measures over days. The model included fixed effects of treatment, day and treatment x day. Differences were considered statistically significant if $P < 0.05$.

Results and Discussion

Effect of probiotics on growth performance, body temperature and fecal pH

Results from Exp. 1 and 2 indicated that supplementation of calf diets with probiotics did not improve the BW gain (P = 0.97 and 0.08 respectively; Fig. 1 and 2; Table 2). The increase in body weight was due to normal growth of the healthy calves as a result of normal feeding. Increasing growth rates across days were reflected in an obvious day effect in both trials (P <0.001 for both). There was no day x treatment interaction when body weight gain was analyzed (P = 0.73 and 0.36 for Exp. 1 and 2, respectively). However, there was a significant difference in average weight gain observed between treatment and control animals in Exp. 2 (P = 0.008) on d 35, which was probably caused by a small number of animals used.

The fecal pH analysis showed no significant day, treatment, nor day x treatment effects (P = 0.83, 0.19 and 0.58, respectively) in Exp. 1 (Fig. 3 and Table 3). When animal rectal temperatures were analyzed, no significant treatment effect was observed in either trial (P = 0.77 and 0.69 for Exp. 1 and 2, respectively; Table 3), but there was a significant day effect (P = 0.02 and P <0.001 for Exp. 1 and 2, respectively) (Fig. 4 and 5). The day effect may be attributed to changes in environmental temperature since the trials were done in summer and environmental temperatures fluctuated, particularly in the second animal trial. There was no day x treatment effect for rectal temperature (P = 0.98 and P = 0.57 for Exp. 1 and 2, respectively).

Effect of probiotic on serum IgA concentrations

Probiotic treatment has been shown to enhance the number of IgA-producing plasma cells in a dose-dependent manner (Perdigon *et al.*, 1995). In the present experiments, serum IgA concentrations were measured. The results showed no significant differences in IgA levels between probiotic treated and control animals (P = 0.61 and 0.30 for Exp. 1 and 2, respectively; Fig. 6 and 7; Table 4). The failure in enhancing secretion of IgA in the serum implied that more than 10^9 CFU.calf^{-1}.d^{-1} might be needed to exert beneficial effects in Holstein calves. Alternatively, longer duration of administration or different strains of probiotics might be needed.

Effect of probiotic on regulation of host immune responsive genes

Although several studies have shown that cytokine production by cells of the immune system can be altered by probiotic use (Tejada-Simon *et al.*, 1999), our results showed that most of the proinflammatory cytokine genes were not upregulated in probiotic-treated animals (data not shown). For example, constant expressions of IL-1β and IL-8 were observed on Days 7 and 35 in both groups of animals (Fig. 10). In fact, this phenomenon was seen across all sampling days, consistent with the findings of Tejada-Simon *et al.* (1999), who reported no effect of repeated oral exposure to viable or killed *L. acidophilus* on basal cytokine mRNA expression in Peyer's Patches, spleen or lymph nodes of mice after 14-day exposure. In contrast, Miettinen *et al.* (1996) showed a strong induction of IL-1β and IL-18 protein secretion when human peripheral mononuclear cells were stimulated with non-pathogenic strains of *Lactobacillus*.

Effect of probiotic on differential counts and phagocytic activity of white blood cells (WBC)

Leukocytes in cattle blood primarily consist of neutrophils, lymphocytes and monocytes. Differential white blood cell count is an important criterion in detection of infection with both viruses and bacteria. Our results indicated that feeding probiotics did not cause a statistical difference in the percentage of lymphocytes (Fig. 11), monocytes (Fig. 12), or neutrophils (Fig. 13), on any sampling day in the first experiment. Consistent with these results, there was no difference in differential WBC counts for lymphocytes except on d 7 (P = 0.037) in Exp. 2 (Fig. 14). Similarly no statistical difference was observed in monocyte counts in Exp. 2 (Fig. 15). However, the percentage of neutrophils in control animals in Exp. 2 was greater (P = 0.005) than in animals whose diets had been supplemented with probiotics (Fig. 16; Table 5). The difference in percentage of neutrophils between the two groups of animals was significant on d 21 (P = 0.0134). Since the animals used in this experiment were healthy and the probiotic used, *L. acidophilus*, is non-pathogenic, a decrease in the percentage of neutrophil counts most likely was due to a small sample size (a total of eleven animals were used in the second experiment).

Furthermore, the phagocytic activity of white blood cells was also measured in both experiments. The results indicated that no difference in phagocytic capacity was observed between control animals and animals treated with probiotics (P = 0.62 and P = 0.53 in Exp. 1 and Exp. 2, respectively; Fig. 17 and Fig. 18; Table 6). Therefore, it can be

concluded that supplementation with the probiotic did not enhance the phagocytic capacity of blood leukocytes.

Collectively, these results revealed no beneficial effect of feeding 10^9 colony-forming units (CFU).calf^{-1}.d^{-1} of *Lactobacillus acidophilus* 381-IL-28 to 3 to 4-month-old Holstein calves on both innate and adaptive immune responses. However, large-scale experiments involving the use of a larger number of animals need to be performed to confirm this conclusion. Moreover, a different dose or strain of probiotic also warrants further investigation.

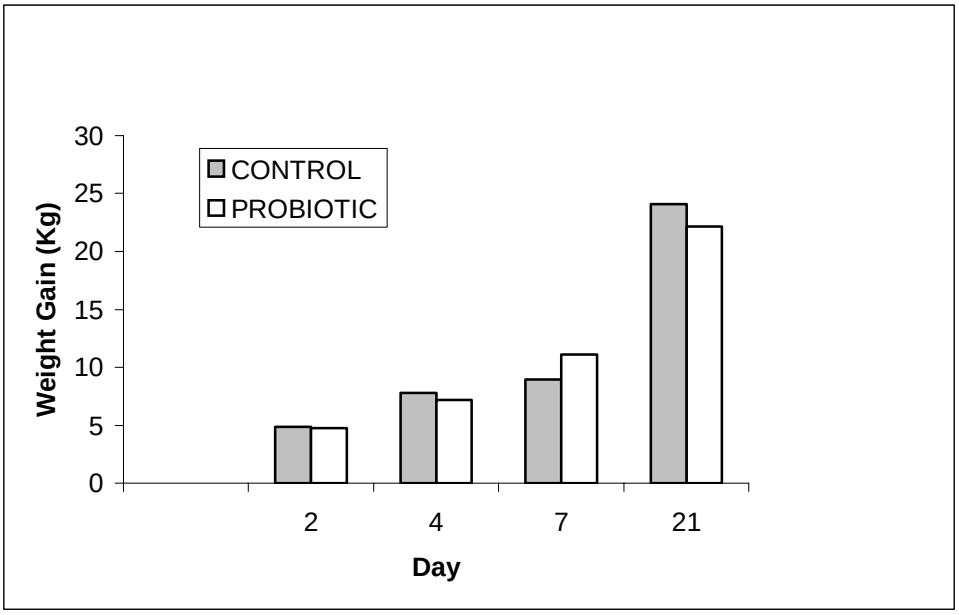

Fig. 1. Effect of probiotic on weight gain of calves in Exp.1. There was a significant day effect ($P < 0.0001$) and no significant treatment ($P = 0.97$) nor day x treatment ($P = 0.73$) effects on weekly weight-gain of the calves. SEM = 2.12

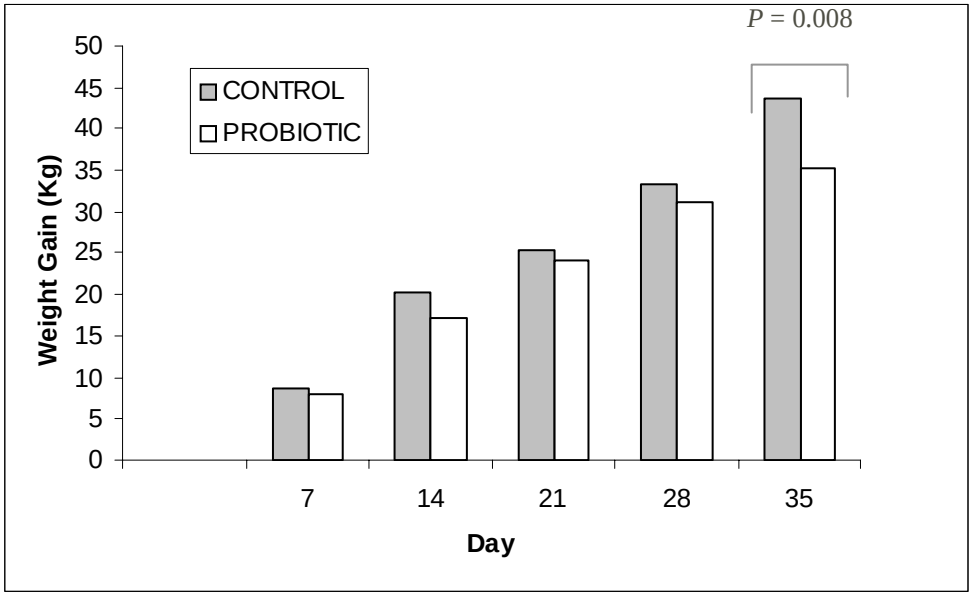

Fig. 2. Effect of probiotics on average calf weekly weight gain in Exp. 2 showing tendency towards a significant treatment effect ($P = 0.08$) with control animals having a higher weekly weight gain in general. There was a significant difference in average weight gain observed on day 35 between the two groups of animals ($P = 0.008$). SEM = 2.4

Table 2. Effect of probiotics on growth performance

Experiment 1				
Item	Control	Probiotic	SEM	*P*-value
n	20	20		
Weight gain (Kg)	10.36	10.31	2.12	0.97
Experiment 2				
Item	Control	Probiotic	SEM	*P*-value
n	30	36		
Weight gain (Kg)	26.22	23.1	2.4	0.08

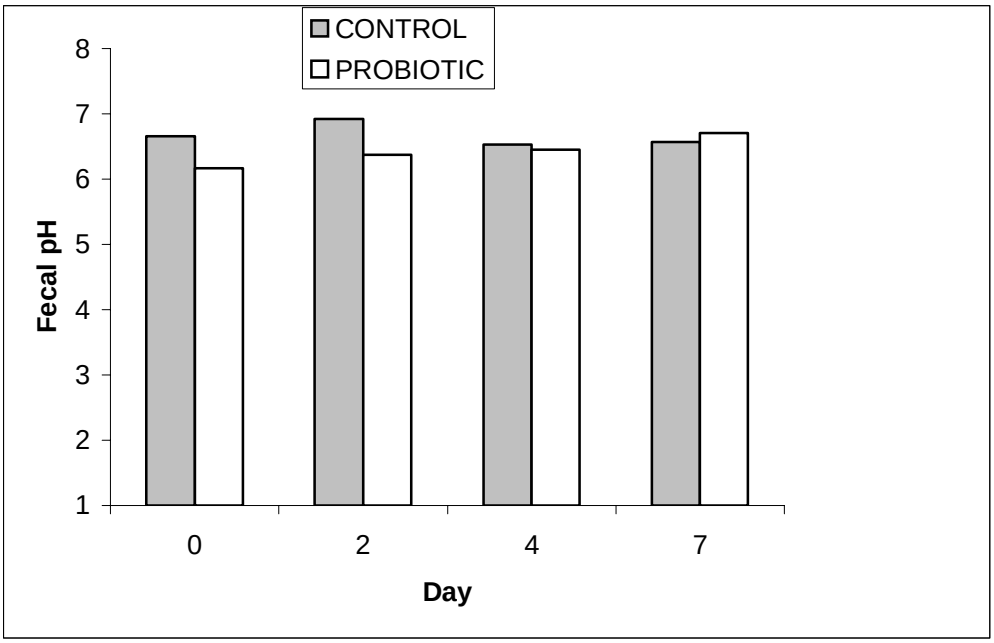

Fig. 3. Effect of probiotics on fecal pH in Exp.1. There was no significant day ($P = 0.83$), treatment ($P = 0.19$) nor day x treatment ($P = 0.58$) effects on fecal pH. SEM = 0.13

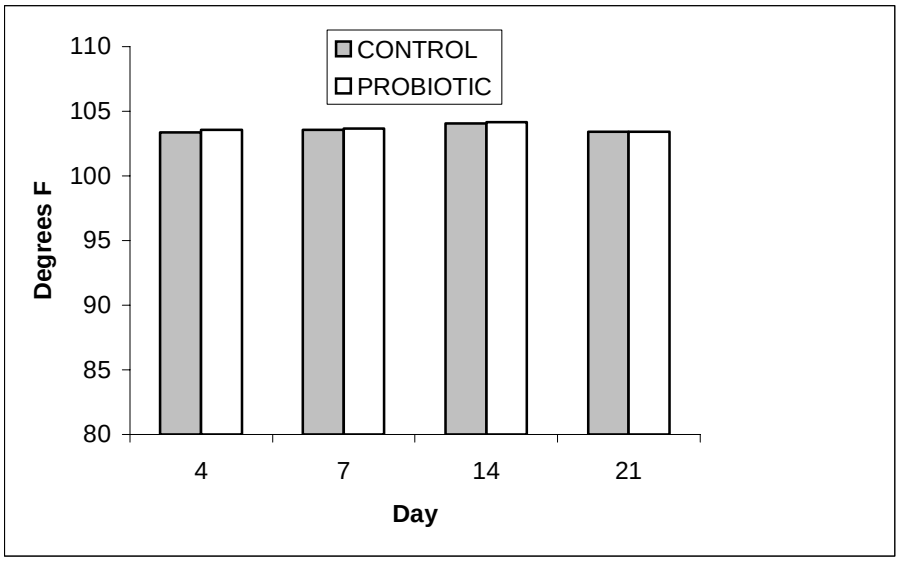

Fig. 4. Effect of probiotics on calf rectal temperatures in Exp. 1. No significant treatment nor day x treatment effects with *P* values of 0.77 and 0.98, respectively. There was a significant day effect (*P* = 0.02) due to fluctuation daily environmental temperature during the experimental period. SEM = 0.24

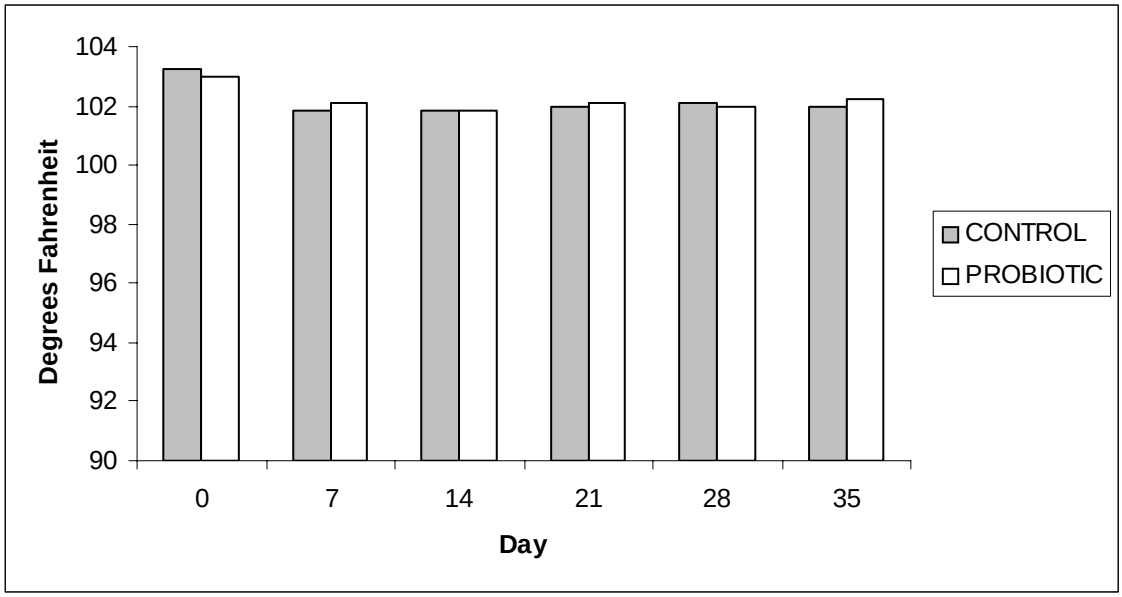

Fig. 5. Effect of probiotics on calf weekly rectal temperatures in Exp. 2. No significant treatment or day x treatment effects with *P* values of 0.69 and 0.57, respectively. There was a significant day effect ($P < 0.001$). Daily environmental temperature varied during the experimental period. SEM = 0.85

Table 3. Effect of probiotics on fecal pH and rectal temperature

Experiment 1				
Item	Control	Probiotic	SEM	*P*-Value
n	16	16		
Fecal pH	6.67	6.42	0.13	0.19
Rectal temp ^{0}F	103.6	103.7	0.24	0.78
Experiment 2				
Item	Control	Probiotic	SEM	*P*-Value
n	30	36		
Rectal temp ^{0}F	102.16	102.21	0.85	0.69

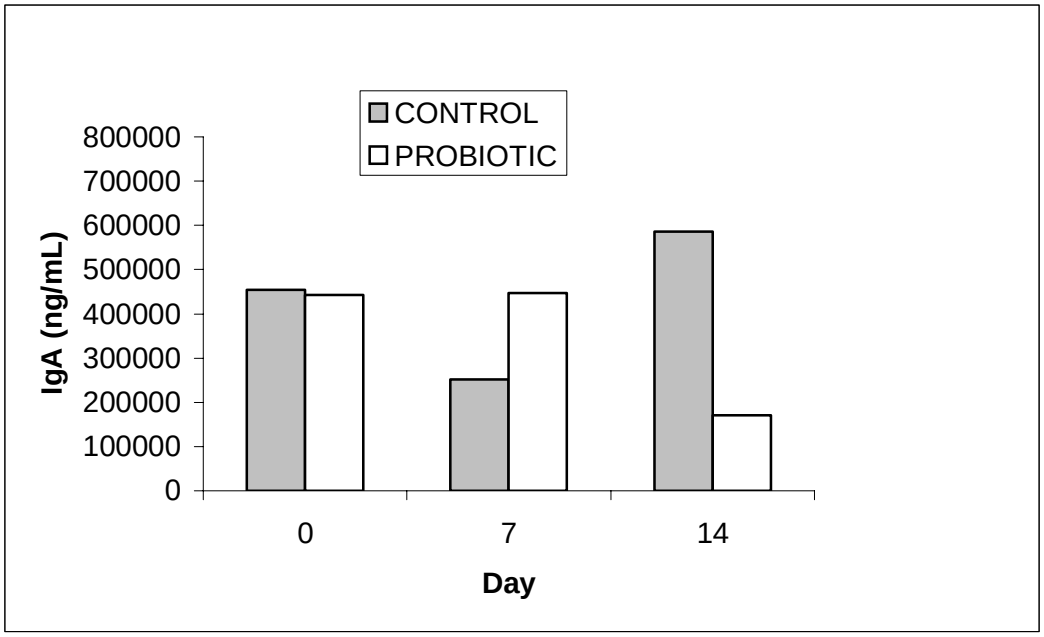

Fig. 6. Effect of probiotics on serum IgA levels in Exp. 1. There was no day *(P* = 0.87), treatment (*P* = 0.61) nor day x treatment (*P* = 0.27) effects on IgA levels. SEM = 1.0 x 10^5

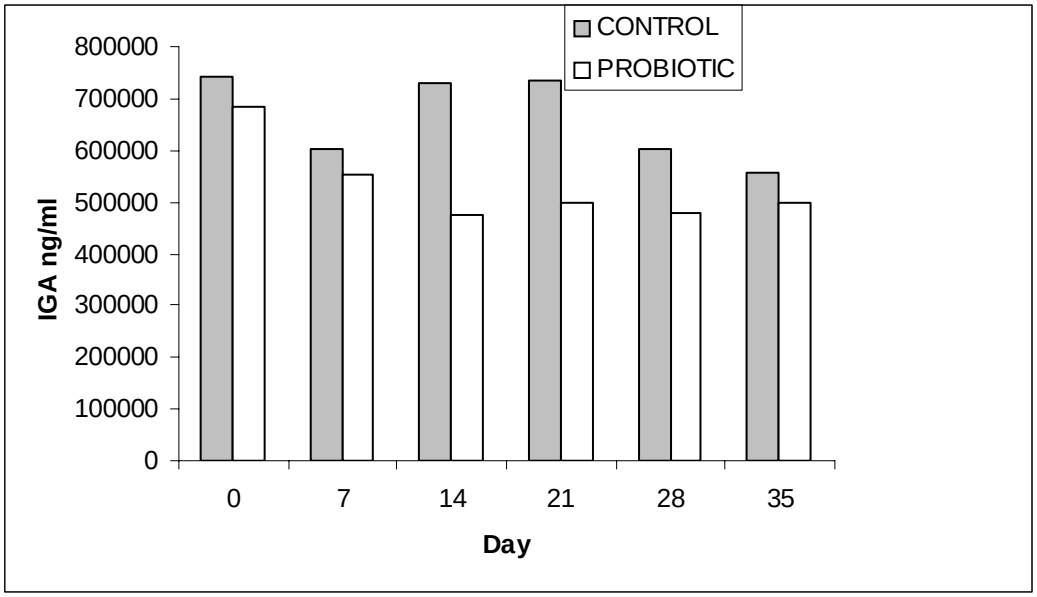

Fig. 7. Effect of probiotics on serum IgA levels in Exp. 2. No significant day (P = 0.47), treatment (P = 0.30) nor day x treatment (P = 0.80) effects were observed between the probiotic-treated and control animals on IgA levels. SEM = 8.7 x 10^4

Table 4. Effect of probiotics on serum concentrations of IgA and IgG

Experiment 1				
Item	Control	Probiotic	SEM	*P*-Value
n	12	12		
IgA (ng/mL)	4.3×10^5	3.4×10^5	1.0×10^5	0.60
Experiment 2				
Item	Control	Probiotic	SEM	*P*-value
n	30	36		
IgA (ng/mL)	6.6×10^5	5.3×10^5	8.7×10^4	0.30

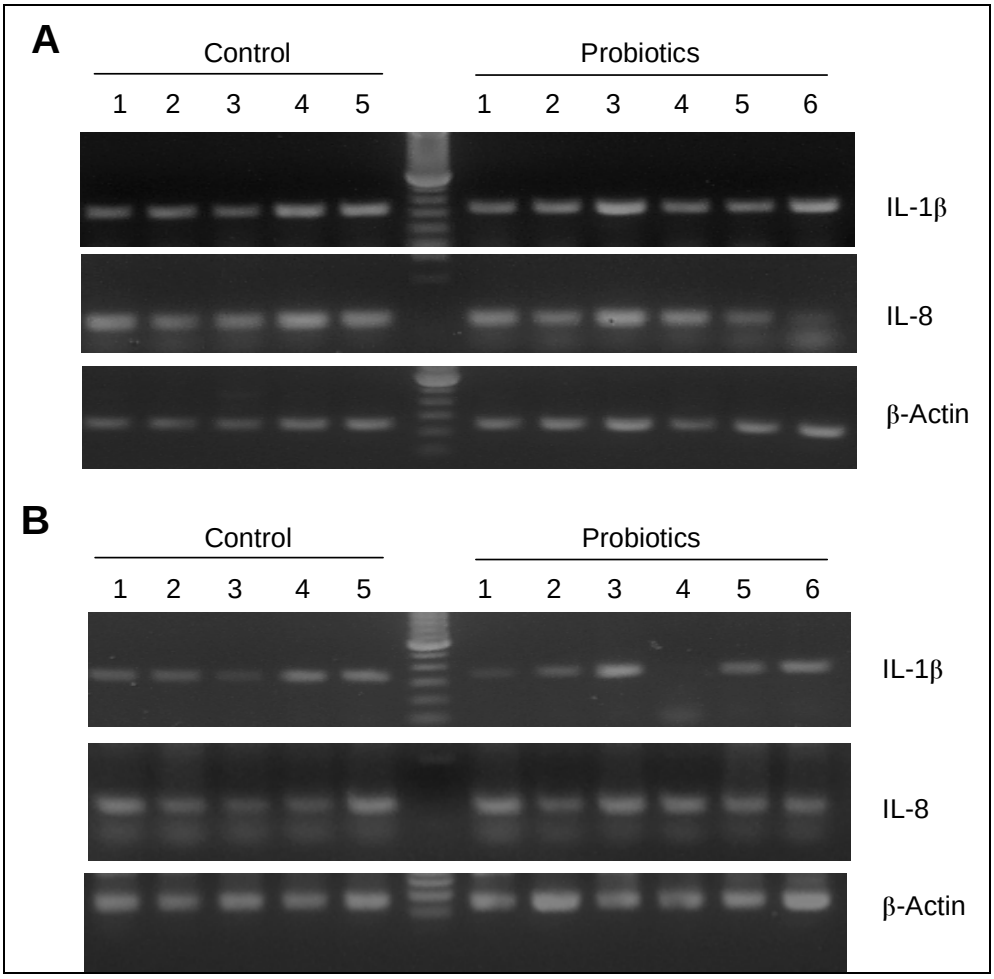

Fig. 8. Effect of probiotic on cytokine gene expression on day 7 (A) and day 35 (B) in Exp. 2. There was no significant difference in cytokine gene expression levels between probiotic-treated and control animals. The cytokine genes, IL-1β and IL-18 were amplified from cDNAs prepared from total RNA isolated from white blood cells prepared from blood samples collected on days 7 and 35, respectively.

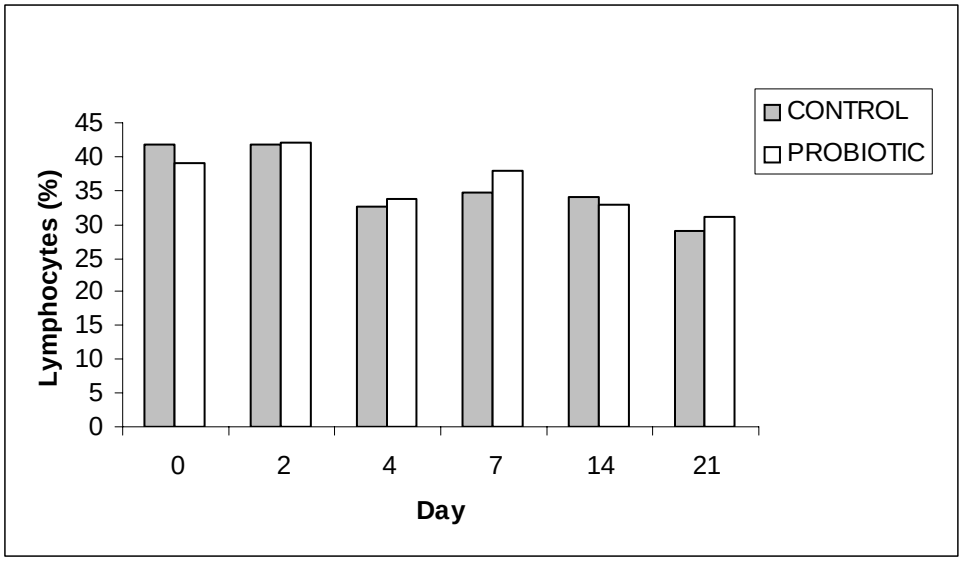

Fig. 9. Effect of probiotics on lymphocytes counts in Exp.1. A significant day (P = 0.04) effect was observed with no significant treatment (P = 0.88) nor day x treatment (P = 0.96) effects on lymphocyte counts. SEM = 1.91

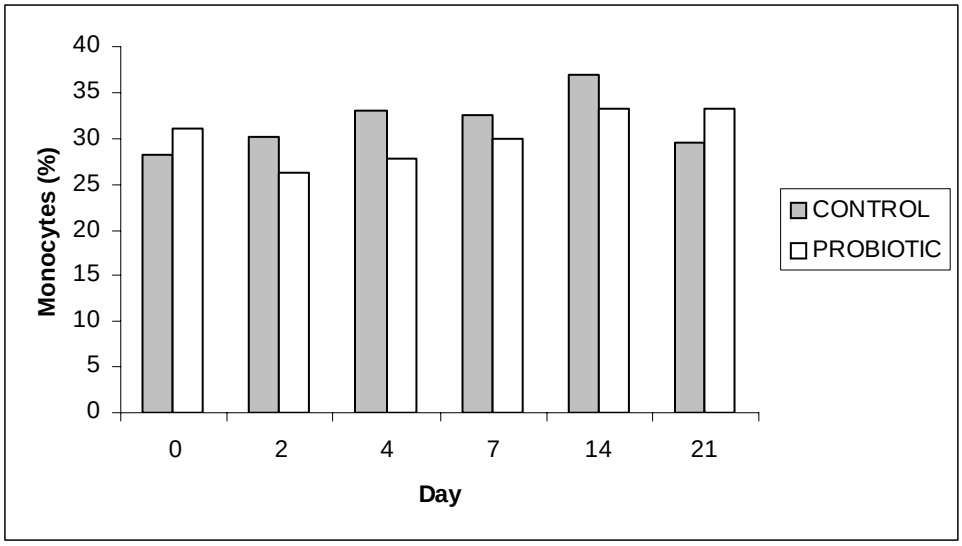

Fig. 10. Effect of probiotic on monocyte counts in Exp. 1. No significant day (P = 0.37), treatment (P = 0.43) nor day x treatment (P = 0.56) effects were observed on monocyte counts between probiotic and control animals. SEM = 1.3

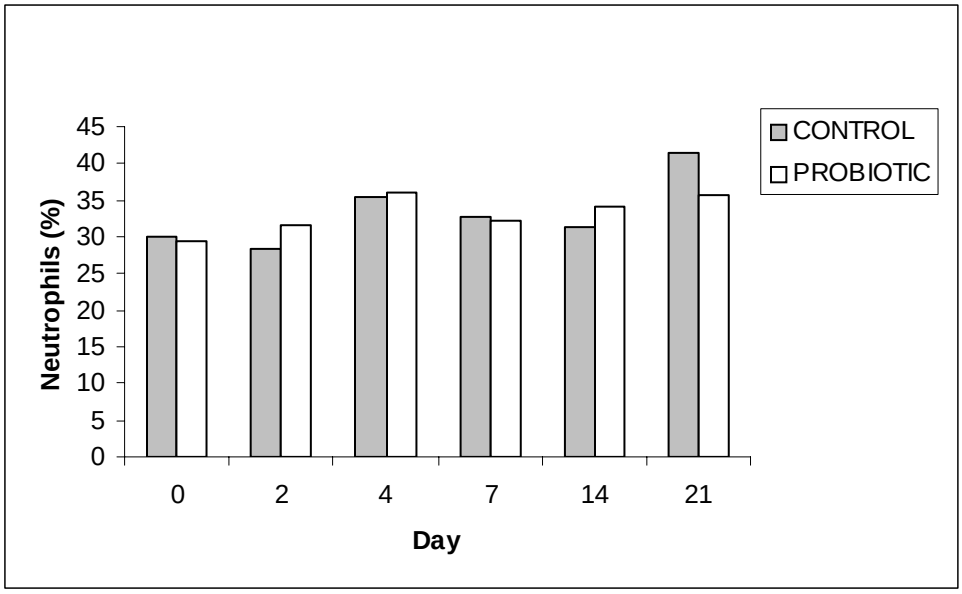

Fig. 11. Effect of probiotic on neutrophils counts in Exp.1. A significant day effect (P = 0.0481) was observed with no significant treatment (P = 0.99) nor day x treatment (P = 0.61) effects on neutrophil counts between probiotic-treated and control animals. SEM = 1.99

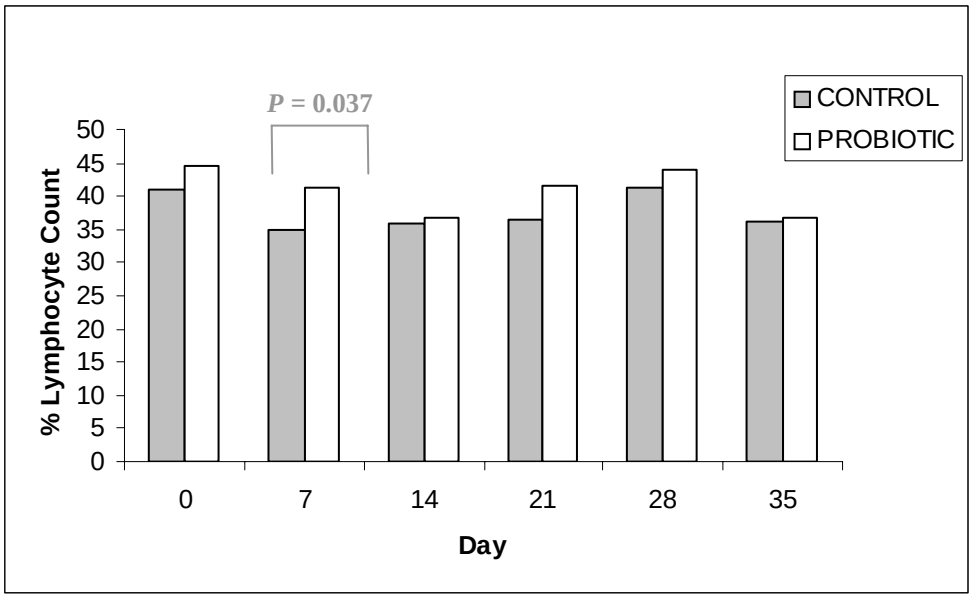

Fig. 12. Effect of probiotic on lymphocyte counts showing significant day (P = 0.009) and treatment (P = 0.024) effects in Exp. 2. The difference in lymphocyte counts between probiotic-treated and control animals was significant on day 7 (P = 0.037). There was no significant day x treatment effect (P= 0.67). SEM = 0.93

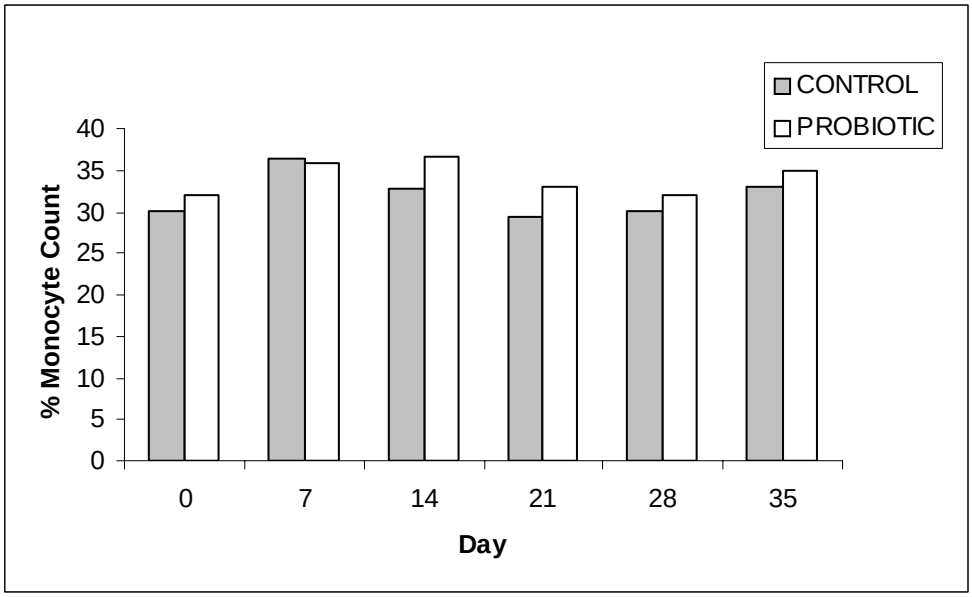

Fig. 13. Effect of probiotic on monocyte counts in Exp. 2; no significant day (P = 0.32), treatment (P = 0.1905) nor day x treatment (P = 0.98) effects were observed. SEM = 1.33

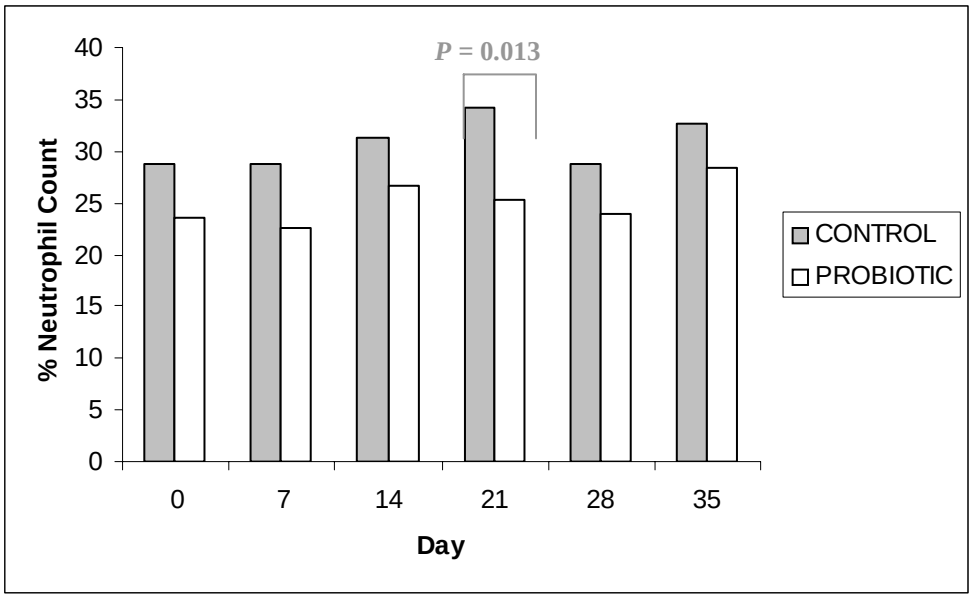

Fig. 14. Effect of probiotic on neutrophil counts in Exp. 2. There was a significant treatment effect (P = 0.052) with control animals showing generally higher neutrophil count. The difference was most significant on day 21 (P = 0.013). SEM = 1.21

Table 5. Effect of probiotics on differential white blood cell count

Experiment 1				
Item	Control	Probiotic	SEM	*P*-value
n	24	24		
% Neutrophils	33.25	33.20	1.99	0.98
% Lymphocytes	35.62	36.04	1.91	0.88
% Monocytes	31.25	30.25	1.30	0.43
Experiment 2				
Item	Control	Probiotic	SEM	*P*-value
n	30	36		
% Neutrophils	30.70	25.00	1.21	0.005
% Lymphocytes	37.56	40.80	0.93	0.02
% Monocytes	31.96	34.11	1.33	0.19

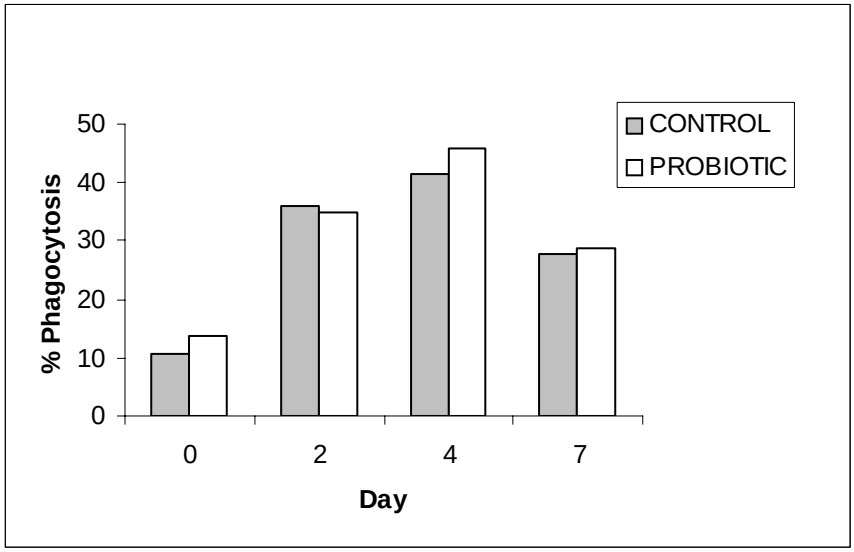

Fig. 15. Effect of probiotic on the phagocytic activity of white blood cells in Exp. 1. There was a significant day ($P < 0.0001$) with no significant treatment ($P = 0.62$) nor day x treatment ($P = 0.82$) effects observed. Therefore, there was no significant difference in phagocytosis capacity between immune cells from probiotic treated and control animals. SEM = 2.5

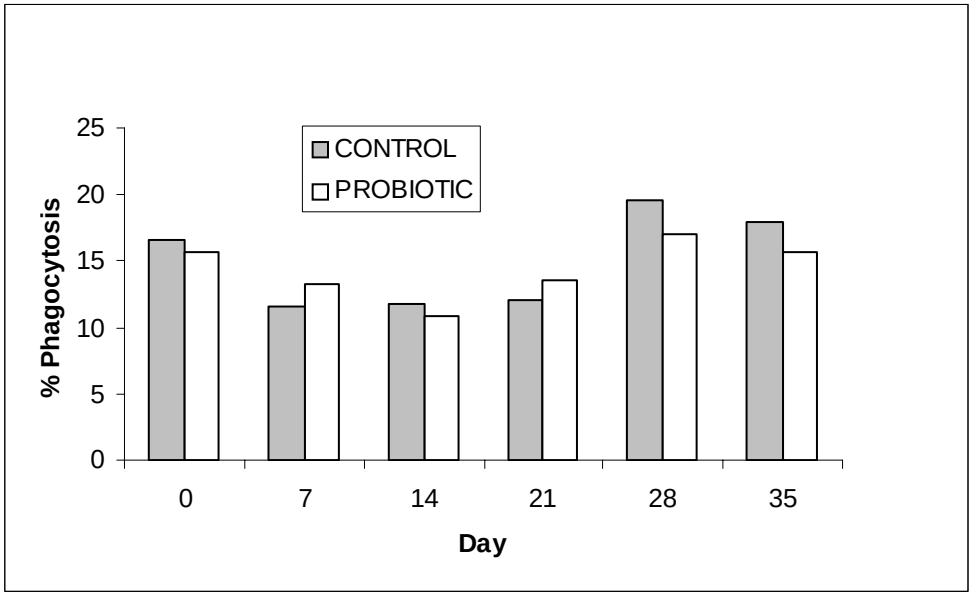

Fig. 16. Effect of probiotic on the phagocytosis capacity of isolated white blood cells from both probiotic treated and control animals in Exp. 2. The results showed neither significant treatment ($P = 0.53$) nor day x treatment ($P = 0.63$) effects. SEM = 0.69

Table 6. Effect of probiotics on phagocytosis of total white blood cells

Experiment 1				
Item	Control	Probiotic	SEM	*P*-value
n	20	20		
% Phagocytosis	28.8	30.7	2.5	0.61
Experiment 2				
Item	Control	Probiotic	SEM	*P*-value
n	30	36		
% Phagocytosis	14.94	14.31	0.69	0.53

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Appendix

Raw data

Exp. 1.

Animal ID	TRT	Day	Phagoc (%)	IgA (ng/ml)	Neu (%)	Lymp (%)	Mono (%)	Wt Gain (Kg)	Temp (^{o}F)	pH
306	PRO	0	12.19	302508	30	38	32			6.55
308	CON	0	9.63	896653	24	41	35			7.13
311	PRO	0	23.68	241466	24	31	43			6.33
307	PRO	0	10.82	1151000	27	47	26			6.13
309	CON	0	9.6	234836	36	42	22			6.5
310	CON	0	17	421333	21	53	26			7.13
303	PRO	0	8.7	75197	37	40	23			5.65
305	CON	0	5.93	265532	39	31	30			5.87
306	PRO	2	23.66		42	38	20	5.9		6.37
308	CON	2	38.4		27	45	28	7.2		6.62
311	PRO	2	33.77		22	49	29	8.2		7.07
307	PRO	2	27.78		26	48	26	1.4		6.67
309	CON	2	34.92		23	50	28	4.5		6.6
310	CON	2	43.1		29	33	38	4.0		6.99
303	PRO	2	54.77		37	33	30	3.6		5.36
305	CON	2	27.45		34	39	27	3.6		7.45
306	PRO	4	54.72		38	22	30	5.9	103.8	6.59
308	CON	4	49.08		42	33	25	6.8	103.2	6.2
311	PRO	4	44.27		41	37	22	9.0	103.4	5.74
307	PRO	4	32.08		29	42	29	5	103.2	6.79
309	CON	4	33.19		28	37	35	6.8	102.5	6.79
310	CON	4	49.26		35	23	42	7.2	103.6	6.76
303	PRO	4	51.81		36	34	30	8.6	103.8	6.69
305	CON	4	33.83		37	37	30	10	104.2	6.37
306	PRO	7	31.3	348166	34	42	24	10.4	103.6	5.58
308	CON	7	38.99	242549	33	42	25	8.6	103.4	6.49
311	PRO	7	30.48	170421	35	37	28	8.6	103.8	7.21
307	PRO	7	23.54	1192000	26	43	31	12.7	103.2	6.81
309	CON	7	19.16	188122	31	35	34	4.5	102.8	6.85
310	CON	7	29.16	311233	29	32	39	10.9	103.2	7.2
303	PRO	7	29.15	75179	34	29	37	12.7	104	7.2
305	CON	7	23.54	265532	38	30	32	11.8	104.8	5.74

306	PRO	14	156441	37	27	36	4.5	105
308	CON	14	1235000	22	45	43	4.5	104.1
311	PRO	14	265537	36	29	35	5.4	103.2
307	PRO	14	187114	28	46	26	8.2	104.3
309	CON	14	302544	43	19	38	1.4	103.1
310	CON	14	453250	25	41	34	9.0	103.4
303	PRO	14	72058	35	29	36	7.2	104.2
305	CON	14	353603	36	31	33	9.5	105.6
306	PRO	21		38	30	32	23.6	103.8
308	CON	21		36	34	30	24	104.1
311	PRO	21		30	28	42	12.3	103
307	PRO	21		26	41	33	22.2	104
309	CON	21		48	25	27	23.6	103
310	CON	21		38	23	39	24.5	103.1
303	PRO	21		49	25	26	30.4	102.9
305	CON	21		44	34	22	24.0	103.5

Exp. 2

Animal ID	TRT	Day	Phagoc (%)	IgA (ng/ml)	Neu (%)	Lymp (%)	Mono (%)	Wt Gain (Kg)	Temp (^{o}F)
291	PRO	0	18.16	607326	40	35	25		102.2
292	CON	0	14.29	575133	22	48	30		103.1
293	PRO	0	14.79	1183570	25	42	33		103.4
295	PRO	0	14.14	886072	26	45	29		102.7
296	CON	0	15.5	776955	21	52	27		105.3
297	PRO	0	19.05	326263	28	46	26		103.3
298	CON	0	19.89	581275	18	36	46		102.8
300	PRO	0	15.55	363652	20	48	32		103.1
301	CON	0	15.31	311331	26	45	29		103.4
302	PRO	0	16.03	780788	20	38	42		102.4
304	CON	0	14.29	1433000	39	37	24		102.7
291	CON	7	13.19	648388	32	38	30	13.2	101.8
292	PRO	7	14.53	468272	19	38	43	10.9	102.1
293	CON	7	11.55	659191	27	43	30	11.3	102.0
295	PRO	7	9.95	1021098	24	31	45	2.3	102.2
296	PRO	7	10.47	522456	27	31	42	5.9	102.3
297	CON	7	18.61	284980	24	47	29	8.6	102.7
298	PRO	7	12.73	410448	24	40	36	6.8	101.9
300	CON	7	13.91	363440	17	44	39	5.4	101.4
301	PRO	7	11.11	632086	30	40	30	3.6	101.4
302	CON	7	10.69	527553	25	45	30	9.0	102.1
304	PRO	7	10.59	803512	31	25	44	14.5	101.9
291	PRO	14	14.95	367404	29	33	38	20.4	101.8
292	CON	14	10.04	437055	28	41	31	19.0	101.6
293	PRO	14	10.85	858738	25	38	37	17.3	102.1

295	PRO	14	4.55	418286	33	28	39	16.8	102.2
296	CON	14	9.835	463843	31	42	27	21.8	101.5
297	PRO	14	10.98	287036	34	37	29	16.4	101.6
298	CON	14	13.97	333732	31	39	30	17.7	102
300	PRO	14	11.4	356144	26	38	36	14.1	101.8
301	CON	14	8.42	734798	26	30	44	16.8	101.9
302	PRO	14	16.93	486797	14	38	48	19.1	101.7
304	CON	14	11.85	1756000	40	35	25	24.1	102
291	PRO	21	16.72	420018	26	42	32	28.2	102
292	CON	21	17.68	421914	22	49	29	25.4	102
293	PRO	21	11.88	605198	28	40	32	17.3	102.2
295	PRO	21	6.815	483226	32	36	32	29.5	102.2
296	CON	21	8.32	506295	41	35	24	19.1	101.9
297	PRO	21	20.38	318792	23	43	34	27.3	102.2
298	CON	21	12.715	572992	33	33	34	23.2	101.8
300	PRO	21	13.74	510004	24	45	31	25.4	102.1
301	CON	21	12.89	631701	31	38	31	27.3	102.3
302	PRO	21	11.26	652467	23	37	40	20	102
304	CON	21	9.936	1531000	40	34	26	28.6	101.8
291	PRO	28	20.74	406822	37	38	25	30.4	102.2
292	CON	28	23.73	613285	22	47	31	30.9	102
293	PRO	28	9.03	553158	26	42	32	28.5	101.9
295	PRO	28	19.92	376734	25	45	30	36.8	101.8
296	CON	28	24.44	412458	22	50	28	37.7	102.4
297	PRO	28	16.43	382365	28	45	27	32.7	102.1
298	CON	28	12.24	724330	18	37	45	27.7	101.6
300	PRO	28	16.93	380614	21	47	32	25.4	102.6
301	CON	28	22.21	675596	27	45	28	35.4	102
302	PRO	28	16.21	575630	22	37	41	31.3	101.6
304	CON	28	18.22	796323	40	36	24	35	102.3
291	PRO	35	23.15	296430	30	32	37	59.1	102.3
292	CON	35	20.59	827270	28	40	32	37.8	102.2
293	PRO	35	16.91	572448	25	38	37	34.1	102.4
295	PRO	35	7.65	424796	33	28	39	42.2	102
296	CON	35	16.43	425028	33	40	27	44.1	101.8
297	PRO	35	15.14	401008	34	37	29	41.8	102.3
298	CON	35	19.18	715597	30	40	30	36.8	102
300	PRO	35	15.62	344833	26	38	36	26.3	102.1
301	CON	35	11.88	682931	30	34	46	38.2	101.4
302	PRO	35	17.69	419971	24	40	36	30	102.4
304	CON	35	18.92	653404	40	35	25	40	102.2

VITAE

Anthony Esheminye Luyai

Candidate for the Degree of

Master of Science

Thesis:	EFFECT OF LACTIC ACID BACTERIA (PROBIOTICS) ON LOCAL AND SYSTEMIC IMMUNE RESPONSES IN CATTLE
Major Field:	Animal science
Personal Data:	Born in Kakamega, Kenya on February 2, 1968, the son of Henry and Nyamatta Luyai.
Education:	Graduated from Alliance High School, Kikuyu in November 1988. Earned Bachelor of Science degree from Kenyatta University, Nairobi, Kenya in 1993. Completed the requirements for the Master of Science Degree with a major in Molecular Immunology at Oklahoma State University in December 2004.
Experience:	Worked as a research technologist at the International Center for insect physiology and Ecology and at the International Livestock Research Institute, in Nairobi, Kenya, in the area of recombinant DNA technology.

Name: Anthony Esheminye Luyai Date of Degree: December, 2004

Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: EFFECT OF LACTIC ACID BACTERIA (PROBIOTICS) ON LOCAL AND SYSTEMIC IMMUNE RESPONSES IN CATTLE

Pages in Study: 68 Candidate for the Degree of Master of Science

Major Field: Animal Science

Scope and Method of Study: The purpose of this study was to investigate effect of probiotics as dietary supplement in three to four month-old Holstein calves on their local and systemic immune responses. Probiotic bacteria are microorganisms that have beneficial effects on the physiology and pathology of their host animals. Their effects on intestinal microflora may play a role in improving animal health. One of their putative effects is the modulation of the immune function. Probiotics have been shown to affect the innate and both the cellular and the humoral arms of the immune system. In this study, *Lactobacillus acidophilus* was administered orally to six calves daily for 35 days at the rate of 10^9 colony-forming units per animal. Five other calves were not given the bacteria and served as controls.

Findings and Conclusions: I observed that there was no significant difference observed in phagocytic activity of immune cells isolated from animals fed on probiotics and the controls. ELISA measurements of serum IgA concentrations of animals fed on probiotics and their controls showed no significant difference. Pro-inflammatory cytokine gene assay, by semi-quantitative RT-PCR also yielded result of no significant difference between the two groups of animals suggesting that the probiotic did not have effect on their regulation. Therefore, I concluded that, at the rate of feeding healthy 3 to 4- month old calves with at least 10^9 colony-forming units of bacteria, no key immunological function is enhanced. Either the bacteria had difficulty in establishing and colonizing the gastrointestinal tract, or the numbers of bacteria fed to the animals were low such that no microfloral balance was created leading to no beneficial effects being conferred to the animals.

ADVISER'S APPROVAL: Dr. Guolong Zhang