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Effects of *Morinda citrifolia* (noni) fruit puree on T cell activation, T cell proliferation and bactericidal activity in neonatal calves during the first two weeks of life

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Abstract

Effects of *Morinda citrifolia* (noni) fruit puree on T cell activation, T cell proliferation and bactericidal activity in neonatal calves during the first two weeks of life

Calves are born with a fully developed but still naïve immune system and are thus vulnerable to high rates of morbidity and mortality. The best defence to ensure calves health and survival is ingestion of colostrum, containing large amounts of maternal immunoglobulins, leukocytes and cytokines. Besides adequate colostrum intake properly used antibiotics can provide much protection for the bovine neonate. However, increased antibiotic scrutiny and in particular consumer demand for organic products have prompted investigations of natural immunomodulators for enhancing calf health. In this context plant-based immunomodulators, such as *Morinda citrifolia* (noni) reportedly having a broad range of immunomodulatory effects, are of noteworthy interest.

The objective of this study was to evaluate the effects of feeding noni fruit puree on the immune system of neonatal calves during the first two weeks of life. For this purpose 18 newborn Holstein Friesian bull calves were acquired in pairs from local dairies. All calves had received 3.785 l (1 gal.) of pooled colostrum by 12 hrs of age. Adequate passive transfer was confirmed at 24 hrs of age. After arrival at the Veterinary Medical Teaching Hospital of the University of Wisconsin in Madison calves were divided randomly into two groups. Group 1 comprised of control calves, while Group 2 received 30 ml (1 oz) of noni puree twice daily in milk replacer. Day 0 samples were obtained before the first feeding of puree between 36 and 48 hrs of age. Peripheral blood mononuclear cells (PBMC) were collected and isolated from each calf on days 0, 3, 7 and 14.

To measure lymphocyte proliferation, a mitogen induced lymphocyte blastogenesis test (LBT) was performed. Mitogen induced activation of $CD4^+$, $CD8^+$ and γδ TCR^+ T cells was evaluated by measuring the up-regulation of the surface protein CD25, which is part of the IL-2 receptor, on these cells with two-color flow cytometry. For both tests Concanavalin A (Con A) and Phytohemagglutinin (PHA) were used as global mitogens. Bactericidal assays were performed to estimate the percent killing of *E. coli* and *Staph. epidermidis.* EDTA anti-coagulated blood was collected from each calf on days 0, 3, 7, and 14.

Results of flow cytometry showed a significant increase in percent CD25 expression on $CD4^+$ and $CD8^+$ T cells on day 3 of the study or approximately 5 days postpartum in response to Con A stimulation. The LBT did not show significant differences between the two groups in response to either mitogen. The bactericidal assay revealed an effect of noni on bactericidal activity against *E. coli*. Noni puree-fed calves had significantly increased *E. coli* bacterial killing when compared to controls on day 14. Differences were not significant on days 0, 3 and 7, but did increase over time. There was no significant difference between groups for *Staph. epidermidis* killing. This immunomodulatory effect may be of high interest for the production animal industry in the future to enhance calves health within the first two weeks of life since antibiotic use becomes more restricted. Additional clinical trials are warranted to investigate the clinical application of noni puree in promoting calf health.

Kurzfassung

Die Wirkung von *Morinda citrifolia* (noni)-fruchtpüree auf die T-Zellaktivierung, T-Zellproliferation und bakterizide Aktivität in neonatalen Kälbern während der ersten zwei Lebenswochen

Kälber werden mit einem voll entwickelten, jedoch aufgrund von nur sehr geringem Antigenkontakt während der Trächtigkeit bis zum Zeitpunkt der Geburt, als naiv zu bezeichnenden Immunsystem geboren. Dieser Umstand hat eine hohe Mortalitäts- und Morbiditätsrate bei neugeborenen Kälbern zur Folge. Die beste Strategie um den Schutz der Neugeborenen zu gewährleisten ist die Aufnahme von Kolostrum, das in großer Menge neben Immunglobulinen auch maternale Leukozyten sowie Zytokine beinhaltet. Darüber hinaus bietet die verantwortungsvolle Anwendung von Antibiotika eine wirksame, wenn auch umstrittene und zunehmend unerwünschte Möglichkeit des Schutzes vor Krankheiten. In diesem Zusammenhang wird immer häufiger der Einsatz von pflanzlichen Immunmodulatoren wie z.B. *Morinda citrifolia* (noni), der man eine Vielzahl von immunmodulierenden Eigenschaften zuspricht, diskutiert und erforscht.

Das Ziel dieser Studie war es den Effekt von *Morinda citrifolia* auf das Immunsystem von Kälbern innerhalb der ersten zwei Lebenswochen zu erforschen. Zu diesem Zweck wurden 18 Holstein Friesian Bullenkälber von mehreren lokalen Farmen bezogen. Nach ihrer Ankunft im Veterinary Medical Teaching Hospital der Univeristy of Wisconsin in Madison wurden die Kälber in 2 Gruppen aufgeteilt. Gruppe 1 diente als Kontrollgruppe, während Gruppe 2 die Tiere umfasste, die während der folgenden 14 Tage zweimal täglich 30 ml Nonifruchtpüree per Milchaustauscher verabreicht bekamen. Zum Zeitpunkt der ersten Blutprobe, die vor der ersten Fütterung des Nonifruchtpürees genommen wurde, waren die Tiere zwischen 36 - 48 Stunden alt. Weitere Blutproben wurden an Tag 3, 7 und 14 gezogen.

Um die Proliferation der isolierten Lymphozyten nach Stimulation mit Con A und PHA zu messen, wurde ein Lymphozytenproliferationstest durchgeführt. Mittels durchflusszytometrischer Untersuchungen wurde die Expression des Oberflächenmarkers CD25, der einen Teil des IL-2 Rezeptors darstellt, nach Stimulation mit Con A und PHA auf den T-Zellsubtypen $CD4^+$, $CD8^+$ and $\gamma\delta$ TCR^+

gemessen. Darüber hinaus wurde ein Test zur Bestimmung der phagozytären Aktivität in Vollblut gegen *E. coli* und *Staph. epidermidis* durchgeführt.

Die Ergebnisse der Durchflusszytometrie zeigten einen signifikanten Anstieg der Expression des Oberflächenmarkers CD25 sowohl auf $CD4^+$ als auch auf $CD8^+$ T-Zellen am dritten Tag der Studie infolge von Stimulation mit Con A. Im Rahmen der Lymphozytenproliferationstests wurden keine signifikanten Erhöhungen der Lymphozytenproliferation infolge der Nonifruchtpüreeaufnahme gemessen. Für die bakterizide Aktivität von Vollblut konnte eine signifikante Erhöhung an Tag 14 gegen *E. coli* festgestellt werden. Außerdem ist zu erwähnen, dass sich die bakterizide Aktivität gegen *E. coli* im Laufe der Studie erhöht hat. Die in dieser Studie beschriebenen immunmodulatorischen Effekte infolge der Fütterung von Nonifruchtpüree an Kälber können besonders im Kontext der ökologischen Herstellung von Fleischprodukten als wichtig angesehen werden. Es sind jedoch noch weitere klinische Studien nötig, um die Mechanismen, die für die beschriebenen Effekte verantwortlich sind, zu erforschen.

1. Introduction

1.1. The mammalian immune system

The mammalian immune system comprises multiple overlapping layers with increasing specificity protecting the host against environmental pathogens such as bacteria and viruses. The first layer of defense, surface barriers like the skin and the surfaces of the gastrointestinal tract, prevents the invasion of the host by pathogenic organisms. The second layer of defense, the innate immunity, responds quickly but non-specific to invading pathogens. The third layer consists of adaptive immune mechanisms. Their specific response to pathogens takes longer compared to innate immune responses, but leads to a long lasting immunological memory. In neonatal calves these mechanisms are fully developed but still immature and newborns are thus susceptible to infections. Therefore, protection provided by colostrum is vitally important during the first days of life until the neonatal immune system has matured. However, adequate passive transfer (APT) often fails and administration of antibiotics is needed. In the following a short overview of the immune system with emphasis on innate and cell-mediated adaptive immunity is given. Afterwards the specifics of the immune system of neonatal calves and the leading strategies of protection, with focus on antibiotics, colostrum and their alternatives, plant-based immunomodulators, are discussed.

1.1.1. Innate immunity

The immediate but non-specific detection followed by the elimination of the invading organisms before they start to proliferate and disseminate in the host by inherent innate immune mechanisms is crucial for an effective immune response. Besides fast recognition and identification of pathogens the immune system must be able to distinguish between self and non-self to spare the tissues of the host. These major functions of the innate immune system mainly depend on leukocytes that originate from myeloid stem cells. These cells can be divided into two large groups. One group comprises mononuclear phagocytes, including macrophages, which derive from monocytes, dendritic cells, and mast cells. The other group

consists of polymorphonuclear phagocytes, such as neutrophils, basophils and eosinophils.

One of the first and most important responses of the innate immune system is inflammation. This reaction of the immune system is triggered either by recognition of invading microbes or by sensing molecules released from damaged tissues or cells through sentinel cells, such as dendritic cells, mast cells and macrophages. For these purposes pattern recognition receptors (PRRs) are expressed on the cell surfaces of a multiplicity of immune cells to detect pathogen-associated microbial patterns (PAMPs). PAMPs are highly conserved molecules that are typically expressed by numerous pathogenic organisms, like for example lipopolysaccharides (LPS) of gram-negative bacteria, lipoteichoic acids of gram-positive bacteria, zymosan yeast particles, lipoproteins of mycobacteria as well as single-stranded bacterial and double-stranded viral ribonucleic acid (RNA) (Pashine et al., 2005). Toll-like receptors (TLRs), as extra cellular receptors, and nucleotide-binding oligomerization domain (NOD) proteins, which are involved in the intracellular recognition of bacteria and their products, are examples for non-phagocytic PRRs. Several different TLRs, each recognizing at least one or a set of specific PAMPs, are expressed on macrophages, mast cells, dendritic cells, eosinophils and epithelial cells of the respiratory tract and the intestine. The toll-like receptor 4, for example, plays a key role in the immune response against gram-negative bacterial infections by recognizing bacterial LPS, which are because of their involvement in the development of septic shock supposedly one of the best known microbial antigens, causing numerous of deaths in veterinary and human medicine every year. LPS binds in the serum to the LPS-binding protein (LBP), which transfers the LPS to the TLR4 co-receptors cluster of differentiation (CD)14 and MD-2 (Kenzel and Henneke, 2006). Binding of CD14 and LPS to TLR4, as well as other PAMPs to their specific TLRs and NODs, initiates a signal transduction cascade leading to the activation of transcription factors such as NF-κB and interferon regulatory factor (IFR) 3. The released cytokines, including the major pro-inflammatory cytokines interleukin (IL)-1, tumor necrosis factor (TNF)-α, IL-12, type 1 interferons (IFN) and chemokines, contribute to the activation, maturation and migration of cells of both innate and adaptive immunity. Other PRRs, such as scavenger receptors (SR), mannose receptors and β-glucan receptors induce phagocytosis.

Pivotal cells of innate immunity are neutrophils and macrophages. Neutrophils, which normally only can be found in the bloodstream, migrate to the site of

microbial invasion attracted by inflammatory mediators (e.g. TNF-α, IL-1, platelet-activating factor (PAF) and eicosanoids). They respond rapidly and are predominately the first immune cells to arrive. Their only task is to kill invading organisms by phagocytosis. Unlike macrophages, they are not capable of sustained phagocytosis. Macrophages arrive after the neutrophils at the site of infection. Besides bacterial products their migration to the site of infection is also triggered by molecules released from damaged tissues and dying cells, such as elastase and collagenase from neutrophils. Their multiple roles include phagocytosis, antigen presentation by major histocompatability complex (MHC) class II molecules to T helper (Th) cells of the adaptive immune system and supervisory functions. Major cytokines, such as IL-1 and TNF-α but also IL-6, IL-12 and IL-18 are released by activated macrophages. The cytokines IL-12 and TNF-α, for example, activate natural killer (NK) cells which start to produce IFN-γ. INF-γ further activates macrophages and thus macrophages are important stimulators of innate and adaptive immunity (Beutler, 2004). Dendritic cells play a crucial role in priming naive $CD4^+$ T helper cells and inducing the differentiation of cytotoxic $CD8^+$ T cells (CTL) by presenting antigen via MHC class II and MHC class I. Immature dendritic cells are activated by recognizing bacterial products, inflammatory mediators and cytokines, such as IL-1 and TNF-α, through their multiple surface receptors.

The major humoral components, which are the second pillar of the innate immune response, are lyzozymes, antimicrobial peptides and the complement system (Rus et al., 2005). The complement system is composed of enzymes and proteins in the blood able to recognize and kill invading microbes. Being part of innate and acquired immunity, it can be activated by mechanisms of both. Three different pathways of activation are known, the classical pathway, the alternative pathway and the lectin pathway. All three pathways lead to the building of a membrane attack complex.

Dysfunction of only one element of innate immunity already evokes a situation of severe immunodeficiency. The antigen-presenting functions of innate immune cells and their production of mediators such as TNF-α, IL-1, IL-12, type I IFNs and CD40L constitute elementary conditions for an effective adaptive immune response. Thus adaptive immunity can be described as subordinate to innate immunity.

1.1.2. Adaptive immunity

Although innate immunity significantly contributes to the defense of the body by an immediate response to invading infectious agents and accomplishing the conditions for an effective adaptive immune response, only the mechanisms of adaptive immunity provide sufficient protection against high levels of disease challenges. In contrast to the innate immune system, which uses predetermined receptors that detect certain structures on the surface of the invading pathogens and therefore being able to respond very quickly, the advantage of the slower developing acquired immunity lies in the ability to learn from every contact, to memorize the acquired information and to generate a targeted immune response. Acquired immunity can be developed either passively by receiving substances such as antibodies or immune cells from an immune animal, which is of particular importance for the protection of newborns, or actively by stimulation through foreign agents such as viruses, bacteria or vaccines. Two different types of acquired immunity can be distinguished: cell-mediated and humoral immunity-antibody-mediated.

1.1.2.1. Cell-mediated immunity- the T cell subsets $CD4^+$, $CD8^+$ and γδ TCR^+

Cell-mediated immunity requires a full repertoire of functional T cells, including $CD4^+$ T helper cells, cytotoxic $CD8^+$ T cells, and γδ TCR^+ subsets. They play a major role in the defense against infectious agents such as bovine herpesvirus-1 (BHV-1) (Endsley et al., 2002), bovine viral diarrhea virus (BVDV) (Endsley et al., 2003; Ridpath et al., 2003) and bovine respiratory syncytial virus (BRSV) (Sandbulte and Roth, 2002; Wollums et al., 2004) threatening young calves. T cells, which derive from the bone marrow and fetal liver, mature in the thymus. In the blood circulation, they account 40 % to 80 % of the lymphocytes. Two populations of T cells can be differentiated by the structure of their T cell antigen receptor (TCR), expressing either α/β or γ/δ chains. All T cells express the surface protein CD3, a signal transducer in the TCR.

T helper cells express CD4 on their surface, which is closely associated to the TCR and thus integrated in the antigen detection. T helper cells are composed of two major populations, T helper 1 (Th1) and T helper 2 (Th2) cells. Whether

activation of naive T cells leads to differentiation into Th1 or Th2 cells depends on a combination of distinct factors. Naive $CD4^+$ T cells are activated by binding antigen-derived peptides presented on MHC class II molecules on antigen presenting cells through their TCR and supporting molecules like CD3. In order to proliferate and differentiate into effector cells, T cell antigen specific receptor- and antigen non-specific costimulation is required. Without costimulation, which is provided by costimulatory molecules on the antigen presenting cells and their ligands on the T cells, such as CD28-CD80 (B7.1), CD40-CD154 and CD27-CD70, T cells undergo apoptosis or become anergic. T cells themselves express molecules, which are responsible for the activation of other cells. For example the binding of CD40L expressed by T cells to CD40 on macrophages activates macrophages. The interaction of CD40 on B cells with CD40L on T cells stimulates the formation of germinal centers by B cells and induces B cells to undergo heavy chain isotype (class) switching and affinity maturation. The interaction of costimulatory molecules between APCs and T cells increases expression of MHC and costimulatory molecules and the production of IL-12 by APCs. IL-12, released by activated dendritic cells or macrophages, in combination with IL-2, induces a Th1 biased immune response. The so-called type 1 cytokines secreted by Th1 cells, including IL-2 and IFN-γ, are responsible for a successful defense against the majority of infections via promoting cellular immune responses such as an early Th1 cell-mediated inflammatory response involving macrophage activation or delayed hypersensitivity reaction (Boehm et al., 1997; Pieters, 2000). Furthermore, IL-12 and IL-18 synergistically stimulate NK cells to produce IFN-γ and thus contribute to the enhancement of microbial activity of macrophages. IFN-γ plays a crucial role in inhibiting the development of IL-4 producing T cells. IL-4, in combination with IL-2, induces the differentiation of Th2 cells, which supports humoral immunity by activating B cells and stimulating antibody production via the secretion of characteristic type 2 cytokines, e.g. IL-4, IL-5 and IL-13. Both types, type 1 and type 2, tend to suppress each other (Brown et al., 1998; Spielberg and Edwards, 2001). It is well established that neonatal immunity favors a Th2 response in the face of an invading pathogen (Levy, 2007). This bias against a Th1 response, during which inflammatory cytokines are produced, reduces the possibility of alloimmune reactions between mother and fetus and helps protect against infection both in- and ex-utero. However, this Th2 biased cell response reduces vaccine responses and leaves the neonate more susceptible to microbial infection (Siegrist, 2000). Furthermore, a high IL-4 expression is correlated with an

increased risk and severity of allergies. Repeated antigen exposure over time, diminishing the Th2 cell polarization and/or increasing the Th1 cell polarization, accelerates the immune maturing process and potentially reduces allergy and atopy (Bach, 2002; Ng et al., 2006). The Th1 immune response, which can be detrimental during pregnancy, becomes increasingly important postpartum as it leads to the production of $CD8^+$ cytotoxic T lymphocytes. Increased secretion of IL-12 and expression of MHC molecules together with IL-2 and IFN-γ from activated Th1 cells stimulate CTLs to respond. Furthermore, activated T cells start to synthesize the α-chain of the interleukin-2 receptor, which increases the affinity of the receptor. Resting T cells express only the β and γ chains of the IL-2 receptor, which results in a lower binding capability of IL-2 (Robb, 1984). IL-2 binding is crucial for initiating T cell growth, proliferation and activation. Helper T cells are not cytotoxic and do not kill or eliminate pathogens directly.

Cytotoxic T cells express CD8 on their surfaces. These cells play a crucial role in the immune system with the ability to attack and lyse infected cells to make invaders become susceptible to humoral immunity (Milon and Louis, 1993; Doherty, 1996; Stenger, 2001; Barry and Bleackley, 2002). Their TCRs recognize processed endogenous antigens on all cells, which are presented by MHC class I molecules. As a result of activation naïve $CD8^+$ T cells proliferate and differentiate into cytotoxic T lymphocytes (CTLs), which secrete IFN-γ, TNF-α and IL-2. If there is no strong immune response or APCs are not infected by the pathogen themselves, $CD8^+$ T cells have to be costimulated by $CD4^+$ Th1 cells. In contrast to T helper ($CD4^+$) cells, where an antigen must be presented in the context of MHC II multiple times simultaneously for more than 48 hrs to a single T helper cell for activation to be induced, just brief presentation, within 24 hrs, of the cognate antigen via a single MHC I molecule is sufficient to activate a $CD8^+$ CTL (Kaech et al., 2002).

γδ T cells make up the largest fraction of PBMCs in young calves (Wyatt et al., 1994; Kampen et al., 2006). They have a wide range of functions, including cytokine production and cytotoxic activity (Welsh at al.; 1997, Boom, 1999; Hensmann and Kwiatkowski, 2001; Passmore et al., 2001), immunomodulation and granuloma organization (Kaufmann et al., 1993; Mombaerts et al., 1993; D´Souza et al., 1997; Smith et al., 1999) and regulation of inflammation (Carding and Egan, 2000). γδ T cells can be divided into different subsets, distinguished by their TCR. One of the subsets expresses the surface molecule Workshop Cluster 1 (WC1). $WC1^+$ γδ T cells are reported to secret IL-12, IFN-γ and TNF-α (Fikri et al., 2001)

and to be capable of antigen presentation to $CD4^+$ T cells (Collins et al., 1998). By secreting the cytokines IL-12 and IFN-γ they promote a Th1 biased immune response.

1.1.2.2. Humoral immunity

The main cells of the humoral immunity are B cells. These cells are defined by the expression of a specific antigen-binding component, the B cell receptor (BCR). Recognition of antigen through the BCR and costimulation activates B cells, which start to divide. The daughter cells become either memory or plasma cells. Plasma cells produce antigen-specific antibodies against the stimulating antigen. Initial exposition leads to a low production of IgM. The interaction with T helper cells and the engagement of CD40-CD40L induces B cells to switch to the production of IgG, IgA or IgE, depending on signals transmitted by the stimulating T helper cells. Memory cells are able to remember prior antigen exposure and are thus able to respond much faster with larger amounts of produced antibodies.

1.2. The immune system of the calf

1.2.1. The development of the immune system of the fetal calf

The development of the immune system of the calf begins early in fetal life. Approximately at day 40 of gestation a fetal thymus is already recognizable. Peripheral blood lymphocytes can be found by day 45. The bone marrow and the spleen appear at day 55 and the lymph nodes by day 60. Bactericidal activity is measurable at day 75 of gestation and hemolytic complement activity by day 90 (Osburn et al., 1982). Neutrophils and macrophages are released to the blood around 130 days and are capable of phagocytosis by late stage gestation (Banks and McGuire, 1989). IgM secreting B cells appear in the circulation already by day 59 whereas IgG B cells do not appear before day 135. Peyer´s patches do not develop before day 175 (Tizard, 2004).

1.2.2. Immaturities of the immune system of the newborn calf

Although newborn calves are capable of mounting a protective immune response, the inherent developmental immaturity of the neonatal immune system represents a predisposing factor towards increased morbidity and mortality. At birth calves move from the sterile environment of the uterus with very limited exposure to antigens into a world full of viral and bacterial pathogens. As a result of the heightened susceptibility to infectious diseases during the first weeks of life, approximately 8.7 % of dairy heifers born alive die prior to weaning (United States Department of Agriculture (USDA), 2002). In Germany average losses in 2006 about 10 % were reported (Over, 2007). Sixty percent of the losses occur in the first week of life, 30 % in the second, mostly caused by diarrhea due to *E. coli* or *Rota-Corona* virus infections. The large number of deaths during the neonatal period causes high financial losses to the dairy industry every year.

The immaturities of the neonatal immune system include a temporarily declined phagocytic activity of neutrophils (Dore et al., 1991, Higuchi et al., 1997) and macrophages at birth as a result of increased steroid levels in the fetal circulation. Menge et al. (1998) reported that neutrophils from neonatal calves have decreased phagocytic activity against *E. coli* when compared to older calves. Sera of the neonatal calves showed insufficient opsonization of bacteria (Lombardo et al., 1979). APCs are reported to have insufficient costimulatory activity (Ridge et al., 1996) and decreased capability of cytokine production (Adkins, 2000). Nonnecke et al. (2003) also reported a decreased cytokine production by blood leukocytes of young calves. PBMCs from 1 week old calves are still functionally hypo-responsive compared to adult PBMCs which is manifested in, for example, a lower production of inducible nitric oxide (NO) and IFN-γ (Rajaraman et al., 1997, 1998). The activity of the classical and the alternative pathway of complement activation are on a lower level than in adults (Mueller et al., 1983). Furthermore, the primary and secondary lymphoid organs are populated by cells which developed independently from antigenic stimulation. Thus the initial response will be a primary response with a prolonged lag period and low antibody concentration.

Besides functional immaturities, composition of the blood leukocyte populations differs considerably from adults as well. Neonatal calves have a higher proportion of γδ TCR^+ cells (Wyatt et al., 1994; Wilson et al., 1996) and lower numbers of circulating B cells, which constitute approximately 30 % of adult levels (Senogles, 1978). According to Kampen et al. (2006) the expression of the B cell maturation

marker CD21 significantly increases over the first 5 months of life until reaching levels comparable to adults. Antibody production activity in suckling calves is low at least up to 1 month after birth (Nagahata et al., 1991; Tizard, 2004).

1.3. Colostrum administration- the leading strategy to reduce morbidity and mortality in neonatal calves

The developmental immaturities of the immune system create a very susceptible situation, especially during the first days of neonatal life which are affected by first contact with large amounts of foreign antigens, as well as by intense organic growth and the establishment of several physiologic functions. The leading management strategy to reduce morbidity and mortality is through the ingestion of high quality colostrum within the first hours of life to ensure adequate passive transfer (APT) of immunoglobulins (Donovan et al., 1998; Weaver et al., 2000; USDA, 2002; Sangild, 2003), given the fact that the absorption of colostral compounds, such as immunoglobulins, colostral cells and cytokines by unselective pinocytosis, initiated through macromolecules, declines within time and ends after approximately 24 hrs postpartum, irrespective of the presence of macromolecules (Besser and Gay, 1985). Although the importance of APT is well known, failures of passive transfer (FPT), mainly standing for an insufficient absorption of immunoglobulins by the calf, occur often (Besser and Gay, 1994). It is assumed that 35 % of dairy calves suffer from FPT (Weaver et al., 2000). Reasons for FPT can be due to the small time window of calves being capable of absorption, time management and feeding method problems of the farmers which might arise more often in larger, commercial calf-raising farms than in smaller farms, as well as poor-quality colostrum (Lee et al., 1983; Quigely and Drewry, 1998). Treatment of FPT comprises, in addition to minimizing the exposure to environmental pathogens, plasma of whole blood transfusion, and oral colostrum supplementation, which exceeds the absorption period, the use of broad-spectrum antibiotics.

1.3.1. Compounds of bovine colostrum

Bovine colostrum contains several substances which are pivotal for the neonatal calf. A very important component of colostrum is the immunoglobulin fraction. The protective role of colostral immunoglobulins, providing calves with passive immunity against infectious disease until their immune systems become fully functional is well established. Although passively received immunoglobulins can inhibit active production of immunoglobulins by the calf (Menanteau-Horta et al., 1985), passive transfer is required for protection, as the syndesmochorial placenta of ruminants totally prevents the trans-placental transfer of immunoglobulins during gestation and consequently calves are born agammaglobulinemic. In addition to immunoglobulins as a source of passive immunity and nutrition, high quality colostrum includes non-immunoglobulin, but immunologically active elements such as a full complement of cytokines, growth factors, hormones and vital maternal leukocytes, which play a central role in modulating both neonatal innate and adaptive immunity (Weaver et al., 2000; Blum, 2003; Reber et al., 2005a, 2005b). Colostral leukocytes are composed mainly of macrophages (40 - 50 %), followed by neutrophils (25 - 37 %) and lymphocytes (22 - 25 %). The lymphocyte populations is comprised of B cells (2.5 - 3.5 %), NK cells (5 - 15 %), and T cells (88 - 89 %) (Duhamel et al., 1987). Liebler-Tenorio et al. (2002) demonstrated uptake and trafficking of colostral leukocytes in the intestinal tract of newborn calves in the epithelium, domes and sinuses around the intestinal lymphoid follicles. Reber et al. (2005) studied the effect of non-cellular colostrum. According to their results, a significant fraction of bovine maternal PBMCs added to acellular colostrum enters the neonatal circulation but leave the vascular compartment within 36 hrs after ingestion and home into neonatal tissues and secondary lymphoid organs.

Bovine colostrum is also known to contain several cytokines, such as IFN-γ, IL-1β, IL-1ra, IL-4, IL-6, IL-18 and TNF-α (Hagiwara et al., 2000; Muneta et al., 2005). Their highest concentrations can be measured immediately after parturition, followed by a rapid decline in milk by five days postpartum (Hagiwara et al., 2000). Yamanaka et al. (2003b) measured the level of the inflammatory cytokines IL-1β, IL-6, TNF-α, and IFN-γ in serum samples taken from neonatal calves within the first 4 weeks of life to determine whether colostral cytokines were absorbed by the neonate. For this purpose samples were taken directly after birth and before the first colostrum uptake. None of the samples contained any of the measured

cytokines. However, they became detectable in the circulation within 12 hrs of the first colostrum feeding and reached peak concentrations between 12 hrs and 24 hrs after birth. During the following weeks, until day 28 postpartum, gradual decreases of cytokines were detectable and finally they were almost undetectable. Hagiwara et al. (2001) supported the concept that cytokines are present and actively absorbed by demonstrating that orally administered recombinant bovine IL-1β appears in the circulation of newborn calves as early as 1 hr after administration and has an immune stimulatory effect through the activation and proliferation of lymphocytes and neutrophils. Similar findings on the transfer of maternal cytokines to the neonate have also been made in neonatal piglets (Nguyen et al., 2007). These studies show that orally administered leukocytes and cytokines are readily absorbed, enter the circulation of the neonate and contribute to the development and maturation of the immune system.

1.4. Antibiotics

Another leading management strategy to reduce morbidity and mortality in neonates, especially in colostrum deprived or deficient calves, is through the administration of antibiotics in milk replacer or by injection as a therapeutic use or prophylactic strategy. In addition to the use for treatment and prevention of diseases antibiotics are used for growth promotion in food animals. While these strategies are associated with reduced calf morbidity and mortality, as well as an improvement of the average daily weight gain and a higher feed efficiency this management technique is highly scrutinized for possibly speeding the development of antibiotic resistant pathogens and resulting in a non-organic product for consumers. The use of antibiotics as growth promoters represents the most continuous use and is therefore the highest risk factor for bacteria developing resistances. The European Union (EU) has already banned the use of antibiotics and related drugs in livestock for the purpose of growth promotion as of January, 1st 2006 (Commission Press Room of the European Union, 2003). Although the risk to human health is, contrary to public assumption, relatively low, these restrictions are intended to preserve antibiotic effectiveness for human use. The World Health Organization (WHO) has also recommended the abandonment of the use of growth-promoting antibiotics in production animals. Denmark was one of

the first countries to ban growth promoters from food animal production. A study published 4 years after the ban demonstrated the positive effect on drug resistant strains in animals and meat. The level of resistance of *Enterococcus* species for example decreased from 60 – 80 % to only 5 – 35 % within this time (Ferber, 2003). The ban exhibited, beside the positive effect of minimizing the risk of resistance, some negative effects on animal health and welfare. Sweden for example, which banned antimicrobial growth promoters in 1996, reported an increase in necrotic enteritis in chickens and colitis in pigs. As a consequence seen in every country affected by the ban, a lowered overall use of antibiotics generates an increased use of therapeutic antibiotics.

Recently a change in consumer habits and demand from cheap, low quality food to high quality, organic food has occurred. This change of habits and increased scrutiny of antibiotics in food animal production has prompted investigation of alternatives to antibiotics. One of the most promising alternatives is the use of immunomodulators to enhance calves health and production, through counteracting stress-induced immune suppression, improving the functions and supporting the development of the immature neonatal immune system.

1.5. Immunomodulators

Immunomodulators, also known as biologic response modifiers (BRM), are substances which are able to influence the immune system by either up- or down-regulating certain parts of the immune response and thereby enhancing the ability of the host to respond faster and adequately to infectious diseases. The effect on the immune system depends on numerous factors, including dose, administration, way and site of action, and the immune status of the host (Tzianabos, 2000). A wide range of immunomodulators, including peptides, such as cytokines and chemokines, lipopolysaccharides, glycoproteins, lipid derivatives, proteins and substances isolated from microorganisms have been identified and tested (Chiang et al., 2003; Schepetkin and Quinn, 2006; Taylor et al., 2006), but only few of them are approved and licensed as immunomodulators by the Food and Drug Administration of the US Department of Agriculture (FDA) for use in food-producing animals. Products such as Immunoboost®, using cell wall fractions of non-pathogenic *Mycobacterium spp.,* ImmunoRegulin®, using heat inactivated

Propionibacterium acnes, or Baypamun® which is also licensed for Europe, using inactivated *Parapoxvirus ovis*, are well established immunomodulatory drugs in veterinary medicine.

Another licensed fast growing group of immunomodulators are nutraceuticals. They are food or ingredients of food having benefits on health by preventing or treating disease. In this context especially probiotics are multiple discussed. Probiotics are non-pathogenic microbes which settle and start to proliferate after ingestion in the intestines. Especially in young animals, having an unstable intestinal flora probiotics can have benefits in promoting health and growth. They build up a protective film by proliferating fast and thus prevent pathogenic agents to settle in the intestines and entering the organism. Moreover, metabolites from probiotic microorganisms can have an antibiotic benefit against pathogens. Besides these protective effects, probiotics can have a positive impact on nutrient uptake (Jeroch et al., 1999). But also polyunsaturated fatty acids, for example, have been used to increase the energy density of animal diets and provide immune modulation (Thanasak et al., 2005). Calves from dams supplemented with mannan oligosaccharides tended to have greater serum rotavirus neutralizing titers and serum protein concentrations compared to control calves (Franklin et al., 2005). Optimal amounts of vitamin A and E have been shown to prompt immune responses similar to that of adult cattle (Rajaraman et al., 1998). However, while having a positive effect on increased neonatal calf growth rate, nutritional supplement by itself has proven to be unsuccessful at increasing adaptive immune response. Furthermore, it actually leads to a reduction in lymphocyte viability (Foote et al., 2007). Cytokines, such as IL-1, IL-2 and IL-6, are also proven to be very effective immunomodulators (Blecha, 2001). Nevertheless, the high production costs make the use of cytokines in food animal production uneconomic. For this reason, especially immunomodulators from botanical sources have gained attention for their broad immune enhancing effects, their relatively low toxicity (Paulsen, 2001; Lewis and Ausubel, 2006; Westendorf et al., 2007) and their bio-availability.

1.5.1. Botanical polysaccharides as immunomodulators

Specifically, botanical polysaccharides obtained from photosynthetic plants, fungi and algae are, similar to bacterial polysaccharides, strong stimulators of the immune system and have a wide array of therapeutic uses, such as immune stimulation (Tzinabos, 2000; Wasser, 2002), anti-tumor therapy (Sergeev et al., 1985; Tzinabos, 2000), wound-healing (Lazareva, 2002), hematopoietic (Gan et al., 1991) and anti-ulceric (Kiyohara et al, 1994).

Important polysaccharides of photosynthetic plants can be found in pectic material or pectin, one of the major cell wall components beside cellulose fibers and hemicellulose filling the interfibrillar space. Major pectic polysaccharides are homogalacturan, rhamnogalacturan-I and rhamnogalcturan-II (Willats et al., 2001). Receptors involved in the recognition of these polysaccharides are pattern recognition receptors (PRR) such as TLRs, SR, β-glucan receptor, CR3, mannose receptor, binding protein of the lectin binding pathway and the alternative and classical complement pathways (Leung et al., 2006). Although these receptors are distributed on several different cell types, myeoloid cells, in particular macrophages, which are one of the most important myeloid cells, are the major stimulatory targets of botanical polysaccharides. Macrophages express a wide variety of receptors (TLRs, SRs, β-glucan receptors and mannose receptors) In response to biologic response modifiers (BRM) stimulated macrophages show enhanced phagocytosis, production of reactive oxygen species (ROS), expression of activation markers like FcR and B-7 molecules and cytokine secretion. Activation of other immune cells can thus be regarded as secondary.

Although polysaccharides are strong stimulators of innate immunity by activating macrophages they do elicit a cell-mediated immune response as well. Acting as T cell independent antigens, they cause a short-lived humoral immune response producing low affinity IgM and some IgG. Thus, most bacterial lipopolysaccharides do not elicit a long-lasting memory and antibody response. Exceptions to these findings are certain microbial and botanical lipopolysaccharides acting as both T cell and APCs activating immunomodulators (Tzinabos, 2000). To date, only a few have been identified and information about structure, function and mechanisms are sparse. One of these BRMs is *Morinda citrifolia* (noni), a well recognized natural herbal product, which is considered to have immune stimulating capabilities mediated by a polysaccharide-rich substance, noni-precipitant (noni-ppt).

1.6. *Morinda citrifolia*

Morinda citrifolia belongs to the botanical family *Rubiaceae*, subfamily *Rubioideae* (Table 1). Within the genus *Morinda* the best known species are *Morinda citrifolia, Morinda trimera* and *Morinda umbellata*. Altogether, there are over 80 species of *Morinda* (Morton, 1992). The species *Morinda citrifolia* can be further divided into the varieties *Morinda citrifolia var. citrifolia*, *Morinda citrifolia var. bracteata* and *Morinda citrifolia cultivar `Potteri`*.

Kingdom:	*Plantae*
Division:	*Magnoliophyta*
Class:	*Magnoliopsida*
Order:	*Gentianales*
Family:	*Rubiaceae*
Genus:	*Morinda*

Table 1. Botanical classification of *Morinda citrifolia*

The scientific name of *Morinda citrifolia* is composed of the Latin words *morus* for mulberry and *indicus* for Indian; Indian mulberry. Several common names such as noni (Hawaii), nono (Cook Islands, Tahiti), kura (Fiji), kudu and cheesefruit, referring to the smell of the ripe fruit, are known. The native distribution of the plant was Southeast Asia and Australia, but currently *Morinda citrifolia* is distributed pantropically between latitudes of 19° North and South (Nelson, 2006).

Noni is an evergreen tree or shrub 3 - 10 m in height, which flowers and fruits all year around. The stem is 13 cm or more in diameter, with a grey or brown, slightly rough bark (Figure 1/A). The opposite leaves, attached by stout petioles 1 - 2 cm long, are of a dark green and shiny colour. They are ovate or elliptic, 14 - 30 cm long by 8 - 18 cm broad with prominent veins. The tubular flowers are five-lobbed, about 6 mm long, and white. The fleshy fruit is greenish-white to pale-yellow, ovoid or globose, 5 - 7 cm long and about 3 - 4 cm in diameter (Figure 1/B). They contain a number of triangular-shaped reddish brown seeds about 4 mm long with an air chamber attached (Figure 1/C). The air sac could be one of the reasons for the world wide distribution of *Morinda citrifolia*. In addition, the long tradition of

use as a medical plant and capability to regain the ability to grow after floating on the water are discussed as other possible reasons for its wide distribution (McClatchey, 2002; Nelson, 2006).

Figure 1/A. Adult noni tree or shrub. Between 3 – 10 m in height.

Figure 1/B. Ripe (yellow) and unripe (green) noni fruits. Dark green shiny leaves with prominent veins.

Figure 1/C. Seeds from the noni fruit surrounded by fruit flesh.

1.6.1. Traditional application of *Morinda citrifolia*

Traditionally the whole noni plant was utilized. The most commonly used parts of the plant are the leaves. The leaves are mainly used either as wraps or chopped for topical treatment of burns and wounds. Poulticed leaves were also used as wraps to treat deep bruising, rheumatism, bone fractures and tuberculosis (McClatchey, 2002; Nelson 2006). Fresh leaves were reported to be used for cooking to wrap and flavour meat or livestock fodder. Tea made from dried leaves was considered to have an analgesic and antipyretic effect. Ripe fruits were, for example, processed as juice or poultice and used as remedy for wounds, abscesses, toothaches, sprains, deep bruising, and rheumatism, but also worm infections. Although the fruits were also consumed as food, their main utilization was famine food (Morton, 1992). Oil obtained from the seed had an insect repellent effect. The wood of the stem was used for building light constructions, such as canoes and paddles (Nelson, 2006). The bark and roots, which contain anthraquinones, were used as red and yellow dyes for clothes and fabrics (Thomson, 1971; Leister, 1975).

1.6.2. Modern application of *Morinda citrifolia*- Research

According to the literature, there is a long list of modern uses of *Morinda citrifolia*. Noni is used because of its antibacterial, anti-tumor, analgesic, hypotensive and immune stimulating effects. In the following paragraph a short overview on the recent research is presented.

Hirazumi and Furusawa (1999) reported that a polysaccharide-rich substance (noni-ppt) obtained from the fruit juice showed anti-tumor activity in the Lewis lung (LLC) peritoneal carcinomatosis model in mice. Furusawa et al. (2003) showed that the same polysaccharide-rich substance has prophylactic and therapeutic potential against Sarcoma 180 ascites tumors in mice. The anti-tumor effect is mediated by an immune stimulatory effect. They have analyzed noni-ppt as a gum arabic heteropolysaccharide substance, composed of the sugars glucuronic acid, galactose, arabinose and rhamnose. Other studies reported a preventive as well as a synergistic effect of Tahitian Noni Juice™ (TNJ) and methylsulfonylmethane (MSM) at the initiation stage of mammary breast

carcinogenesis induced by 7,12-dimethylbenzo(α)anthracene (DMBA) in female SD rats (Wang et al., 2002, 2003). Liu et al. (2001) reported an inhibitory effect on the AP-1 transactivation and cell transformation in the mouse epidermal JB6 cell line of glycoside found in the fruit of the Noni tree. Sang et al. (2003) showed an inhibitory effect on UVB induced transcriptional AP-1 activity of iridoids from the leaves. An anti-inflammatory effect was reported by Xu et al. (2006), who showed that foals orally fed 60 ml of noni juice twice a day had an immense reduction in COX-2, TNF-α, IL-1β, IL-8 and IL-6 mRNA expression in LPS-stimulated monocytes.

1.6.3. Pharmacological ingredients of *Morinda citrifolia*

Despite the numerous traditional therapeutic uses and ongoing research, little information about the biologically active components is available. The following information on the phytochemistry of *Morinda citrifolia* has been acquired from several published articles.

The fruits and the leaves of the *Morinda citrifolia* tree reportedly contain numerous iridoids, such as asperuloside, asperulosidic acid and deacteylasperulosidic acid (Levand and Larson, 1979; Sang et al., 2002; Kamiya et al., 2005). Furthermore, flavonol glycosides and triterpenes were found in the leaves (Sang et al., 2002). According to a study in 2006 by Sang et al., a wide spectrum of anthraquinones in the roots, including rubiadin, damnacanthal and alizarin-1-methyl ether was found. The fruits contain polysaccharides, fatty acid glycosides, anthraquinones, coumarins such as scopoletin (Pawlus et al., 2005), flavonoids, ligans, phytosterols, carotinoids, monoterpenes, short chain fatty acids and fatty acid esters (Potterat and Hamburger, 2007). The major polysaccharides, which can be found in the noni fruit comprise of homogalacturonans, rhamnogalacturonans I, arabinans, and type I and II arabinogalactans (Bui et al., 2006).

2. Materials and methods

The objective of this study was to evaluate the effects of *Morinda citrifolia* fruit puree on the immunity of neonatal calves in the first two weeks of life. Therefore, a bactericidal assay was performed to estimate the effect of noni on gram-positive and gram-negative bacterial killing. Activation of T cell subsets in response to Concanavalin A (Con A) and Phytohemaglutinin (PHA), lectins obtained from the jack bean (*Canavalis ensiformis*) and the red kidney bean (*Phaseolus vulgaris*) respectively, was measured by two-color flow cytometry analysis of CD25 expression, the α-chain of the IL-2 receptor, on T cell subsets $CD4^+$, $CD8^+$ and γδ TCR^+. Resting T cells express only the β and γ chain of the IL-2 receptor (Robb, 1984, Waters et al, 2003). In addition to the activation of T cell subsets lymphocyte proliferation in response Con A and PHA was measured performing a lymphocyte blastogenesis test (LBT) (Sandbulte and Roth, 2004).

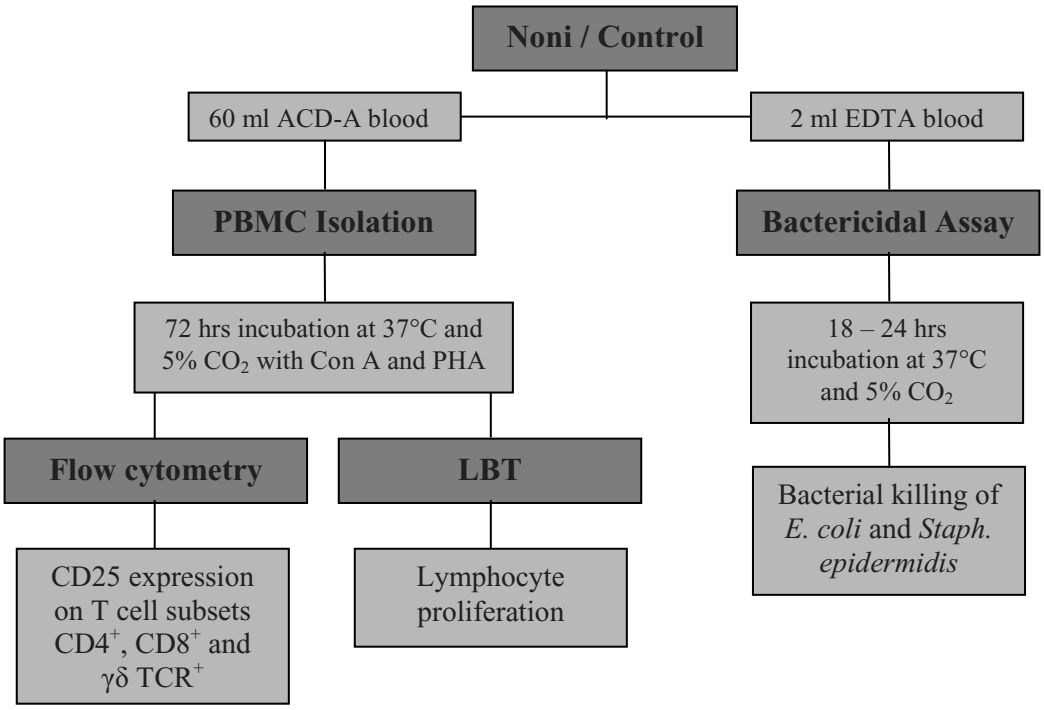

Figure 2. Experimental design

2.1. Reagents

The following reagents were prepared in the laboratory of the Veterinary Medical Teaching Hospital of the University of Wisconsin-Madison:

a) Sterile anticoagulant Acid Citrate Dextrose-A (ACD-A) was prepared with 2.2 g sodium citrate (dehydrate), 0.8 g citric acid (monohydrate), 2.5 g dextrose and 100 ml H_2O q.s.

b) EDTA (Promega Corp., Madison, WI, USA) was prepared as 2 mM stock solution in 1x Dulbecco´s phosphate buffered saline (D-PBS) without Ca^{++} and Mg^{++} and phenol red (Gibco, Invitrogen Corp., Carlsbad, CA, USA).

c) MCM solution was prepared with 250 ml Roswell Park Memorial Institute Media (RPMI) 1640 (Mediatech, Inc. Herndon, VA, USA) containing 2.05 mM L-glutamine but no phenol red (Invitrogen, Carlsbad, California, USA), 50 ml 10 % heat inactivated fetal calf serum (FCS), 5 ml glutamine and 5 ml Penicillin / Streptomycin.

d) Cell lysing solution of pH 7.2 was prepared by dissolving 1.5 g (10.6 mM) Na_2HPO_4 and 0.32 g (2.7 mM) NaH_2PO_4 in 1 L H_2O q.s.

e) Restoring solution of pH 7.2 was similarly prepared by dissolving 1.5 g (10.6 mM) Na_2HPO_4, 0.32 g (2.7 mM) NaH_2PO_4 and 27 g (462.0 mM) NaCl in 1 l H_2O q.s.

2.2. Animals and experimental design

The study and procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of University of Wisconsin-Madison. Eighteen newborn Holstein bull calves were acquired from 6 local dairies over a 4 month time period. The animals arrived in pairs within 12 hrs after birth at the Veterinary Medical Teaching Hospital of the University of Wisconsin in Madison where they were housed individually in pens on sawdust. Stalls were cleaned twice

a day. It was assured that every calf had received 3.785 l (1 gal.) of pooled colostrum by 12 hrs of age. After arrival, every animal was subject to a physical examination, followed by daily examinations by internists boarded in the American College of Veterinary Internal Medicine blinded to treatment groups, including temperature, ease of cough induction, fecal consistency, and presence and severity of ocular or otic abnormalities and the navel was dipped in iodine. Results of the examinations were recorded as calf health scores (Table 3). Any calf receiving a total health score of ≥ 5 for 3 consecutive days would have been excluded from the study and treated by a veterinarian. No calf had to be removed. Total Protein (TP) and Packed Cell Volume (PCV) were also measured after arrival in ethylenediaminetetraaceticacid (EDTA) anticoagulated blood samples taken from the jugular vein. All values were within the normal range (Table 2). At 24 hrs of age a Plasma Calf IgG Midland Quick Test Kit® (Midland Bioproducts Corporation®, Boone, IA, USA) was used to confirm APT (IgG >1000 mg / dl) of immunoglobulins via colostrum for all calves (Tyler at al., 1996). To measure the IgG concentrations in newborn calves EDTA anticoagulated blood was collected from the jugular vein and 200 µl of whole blood were transferred into the dilution vial. After mixing the sample thoroughly, the cassette sample well was completely filled with the dilution mixture. Results were read between 20 and 40 min after placing the dilution mixture in the cassette sample well. All animals had levels over 1000 mg / dl, which indicates an adequate passive transfer of immunoglobulins.

Pair	**Body Weight** (lbs)		**Total Protein** (g / dl)		**Packed Cell Volume** (%)		**IgG concentration**
1	110	98	7.2	6.8	33	36	> 1000 mg / dl
2	89	96	6.9	6.3	34	38	> 1000 mg / dl
3	103	105	6.9	7.0	35	34	> 1000 mg / dl
4	112	89	6.7	6.5	37	39	> 1000 mg / dl
5	108	106	6.6	7.4	38	32	> 1000 mg / dl
6	99	105	6.4	6.7	34	35	> 1000 mg / dl
7	120	101	6.9	6.2	38	37	> 1000 mg / dl
8	96	93	7.1	6.6	36	32	> 1000 mg / dl
9	100	107	6.8	6.8	33	35	> 1000 mg / dl

Table 2. Body weight , Total Protein, PCV and IgG concentration after arrival at the VMTH

The calves were randomly divided into 2 groups: group 1 represents non-treated control calves; group 2 is comprised of the noni treated animals. The day after arrival at the Veterinary Medical Teaching Hospital was designated day 0. A baseline blood sample was drawn on day 0, prior to the first noni feeding, at which time the calves were 36 - 48 hrs old. Further blood samples were taken on days 3, 7, and 14. The animals were bottle-fed 2.28 l (2 qts) of milk replacer (CALF GLO® Insta Mix Milk Replacer, Vita Plus Corporation) twice a day for the first 7 days and 2.85 l (2.5 qts) from day 8 to 14 by technicians of the VMTH. They also had free access to 207 ml (7 oz) of calf starter (18 % High Energy Calf Starter – RUM, Middleton Farmer's Co-op) and 3.785 l (1 gal) of water per day. The calves in group 2 received 29.57 ml (1 oz) of noni twice a day in their milk replacer. At the end of the study calves were sold.

	Temperature	Cough	Nasal Discharge	Eyes or ears	Fecal Score
0	37.8-38.3°C	No cough inducible	Normal serous discharge	Normal	Normal
1	38.4-38.8°C	Induced single cough	Small amount of unilateral, cloudy discharge	Small amount of ocular discharge	Semi-formed, pasty
2	38.9-39.4°C	Induced repeated cough or occasional spontaneous cough	Bilateral, cloudy or excessive mucus discharge	Moderate ocular discharge for both eyes or slight ear drop	Loose but enough consistency to stay on bedding
3	$\geq$ 39.5°C	Repeated spontaneous coughing	Copious, bilateral, mucopurulent nasal discharge	Head tilt or both ears dropped	Watery, sifts through bedding

Table 3. Calf health scoring criteria developed by Dr. Sheila McGuirk from the University of Wisconsin in Madison

2.3. Blood collection

Before taking the blood samples a large area around the puncture was cleaned with alcohol and gauze. 60 ml of blood, which was used for flow cytometry analysis and LBT, was collected from the jugular vein of each calf on days 0, 3, 7 and 14. The first samples were obtained from each calf between 36 and 48 hrs of age, before the first feeding with noni, which constituted day 0. The samples were

collected into four 15 ml vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ, USA) containing 15 % ACD-A. An additional 2 ml blood sample for the bactericidal assay was collected into 2 ml K2 EDTA vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ, USA) (Figure 3). The blood samples were processed within 1 hr of collection.

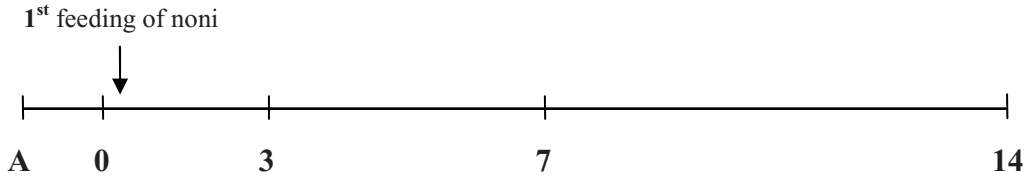

Figure 3. Timetable of the study. A = arrival at the VTMH.of the calves between 36 - 48 hrs of age. Samples were taken at days 0, 3, 7, and 14.

2.4. Isolation of peripheral blood mononuclear cells

PBMCs were isolated by density gradient centrifugation using 1.083 Ficoll-Histopaque (Sigma-Aldrich, St. Louis, MO, USA) in combination with hypoosmotic erythrocyte lysis.

The 60 ml ACD-A anticoagulated blood samples were divided into two sterile 50 ml conical centrifuge tubes (Becton Dickinson, Franklin Lakes, NJ, USA), 25 ml per tube. The sample was diluted 1:1 with D-PBS without Ca^{++}, Mg^{++} and phenol red. The diluted samples were centrifuged at 920 x g for 30 min at room temperature to extract the buffy coat, which was harvested carefully afterwards and filled up to 15 ml with plasma for flow cytometry and LBT. 15 ml of cell dilution were layered carefully onto 10 ml 1.083 Ficoll-Histopaque and centrifuged at 1380 x g for 30 min at room temperature. Due to the higher density, erythrocytes and granulocytes were accumulated at the bottom of the tube. The interphase, located between the plasma and the separation medium, contained PBMCs and lymphocytes. In our study some erythrocytes were found in the interphase, which presumably was caused by the young age of the calves. After the interphase was harvested carefully 20 ml lysing solution were added to lyse the remaining erythrocytes. After 1 min the lysing process was stopped by neutralizing the lysing solution with 10 ml restoring solution. This step was followed by centrifugation at

280 x g for10 min. Afterwards cells were washed twice at the same speed and time with 25 ml PBS and with 25 ml pre-warmed RMPI 1640. After the last washing step, cells were resuspended in pre-warmed RPMI 1640 with 20 % FCS for flow cytometry and LBT. Viability of cells, determined by Trypan blue exclusion dye, always exceeded 95 %. To get a concentration of 5 * 10^6 cells / ml. for flow cytometry analysis and LBT cell counts were performed using a light optical microscope.

2.5. Bactericidal assay

The purpose of this modified *in vitro* bactericidal assay is to estimate the effect of noni fruit puree on the ability of phagocytic cells to engulf and kill bacteria.

For the bactericidal assay blood samples were collected in 2 ml K2 EDTA vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ, USA) from the jugular vein of each calf on days 0, 3, 7 and 14. Day 0 samples were obtained from each calf between 36 and 48 hrs of age and before the first feeding of puree. Bacteria, *E. coli* and *Staph. epidermidis* isolates, were obtained from calves with clinical cases of septicaemia and mastitis respectively. These isolates were inoculated onto trypticase soy agar with 5 % sheep blood (BBL, Becton Dickinson, Sparks, MD) and incubated at 37°C for 18 - 24 hrs. For the bactericidal assay, colonies of each bacterium were resuspended in tryptic soy broth (TSB). The number of bacteria was determined by a 0.5 McFarland standard at 1.5 x 10^8 cfu / ml. Each sample consisted of 450 µl blood added to 500 µl RPMI 1640 media plus 50 µl bacteria. Bacterial growth controls were prepared by adding 50 µl diluted bacteria to 950 µl RPMI 1640. After 2 hrs incubation at 37°C of the experimental samples and bacterial controls ten fold serial dilutions in TSB of the incubated samples and controls were made. Dilutions were plated in duplicate on trypticase soy agar with 5 % sheep blood and incubated overnight at 37°C. Only plates with 30 and 300 colonies were counted. Before calculating percentage of bacterial killing dilution was adjusted and colony forming units per ml (cfu / ml) were determined (Koch, 1981; Wallis et al 2001). Numbers of cfu´s were averaged, if both plates contained between 30 and 300 colonies.

$$\frac{(\text{positive control cfu/ ml} - \text{sample cfu/ ml})}{\text{positive control cfu/ ml}} \times 100 = \text{percent bacteria killed.}$$

2.6. Flow cytometry

Flow cytometry uses the principles of light scattering, light excitation and emission of fluochrome molecules to generate multiparameter data. This method allows an identification of cell immunophenotypes and measurement of cell surface protein expression by using fluorescent-conjugated antibodies with different emission spectra.

For flow cytometry experiments PBMCs were cultured on a Costar flat bottom tissue culture treated 96 well plates (Corning, Inc., Corning, NY, USA). Per calf 42 wells were prepared as described below:

a) 12 wells were prepared with 100 µl RPMI 1640 + 20 % FCS (unstimulated controls)
b) 21 wells were prepared with 100 µl of Con A (final concentration of 6.7 µg / ml; Sigma, St. Louis, MO, USA
c) 9 wells were prepared with 100 µl of PHA M form (Gibco, Invitrogen Corp., Carlsbad, CA, USA) diluted 1:200 in RPMI 1640
d) 50 µl / well cell suspension with a concentration of 5 * 10^6 cells / ml were added to all 42 wells

No Mitogen	Con A	PHA
CD4 + CD25	CD4 + CD25	CD4 + CD25
CD8 + CD25	CD8 + CD25	CD8 + CD25
TcR1-N6 + CD25	TcR1-N6 + CD25	TcR1-N6 + CD25
Cells only		

Table 4. Antibody- mitogen staining combinations used for two-color flow cytometry analysis

After incubation for 72 hrs at 37° C and 5 % CO_2, cells were harvested and three identical wells were transferred into a 12 x 75 mm polystyrene round bottom tube (Becton Dickinson, Franklin Lakes, NJ, USA). For washing, 1 ml D-PBS was added and centrifuged at 520 x g for 7 min at 25°C. After centrifugation, the supernatant was discarded, cells resuspended and stained for two color flow

cytometry with 50 µl of the primary mouse α-bovine antibodies (VMRD, Pullman, Washington, USA) at a concentration of 15 µg / ml as specified in Table 5.

Antibody	Immunglobulin class	Catalog number
CD2	IgG_{2a}	MSA4
CD4	IgM	GC50A1
CD4	IgM	DH29A
CD8α	IgM	BAQ111A
TcR1-N6[1)]	IgM	CACTB6A
CD25	IgG_{2a}	CACT108A

Table 5. Primary mouse α-bovine antibodies. [1)] γδ T lymphocyte subpopulation

Stained cells were incubated on a shaker for 20 min at 4°C and washed 3 times by adding 1 ml PBS and centrifugation at 520 x g for 7 min at 21°C. After the last washing step, the supernatant was discarded; cells resuspended and stained with 50 µl of the 1:200 diluted secondary antibodies per tube. The secondary antibodies (Table 6) were acquired from Jackson ImmunoResearch (Westgrove, PA, USA). The fluorescein-conjugated antibodies were used for staining $CD4^+$, $CD8^+$, γδ TCR^+, $CD3^+$ T cells and isoptype control. R-Phycoerythrin-conjugated antibodies were used to detect the CD25 expression.

Antibody	Catalog number
Fluorescein (FITC)-conjugated AffiniPure Fab Fragment Goat Anti-Mouse IgM, µ Chain Specific	115-097-020
R- Phycoerythrin (PE)-conjugated AffiniPure Goat Anti-Mouse IgG, Fc Subclass 2a Specific	115-115-206
Fluorescein (FITC)-conjugated AffiniPure Goat Anti-Mouse IgG, Fc subclass 1 Specific	115-095-205

Table 6. Secondary antibodies used for two-color flow cytometry

After 20 min incubation on a shaker at 4°C, cells were centrifuged and washed 3 times as before. After the last washing step, the cells were resuspended in 500 µl PBS containing 1 % paraformaldehyde. A two-color flow analysis was performed 15 min after cell fixation. A Becton Dickinson FACScan flow cytometer was used, and the results were analyzed in FlowJo (Ver. 8.1.1: Tree Star, Inc., 1997-2006).

2.7. Lymphocyte blastogenesis test

The Lymphocyte Blastogenesis assay is a well established method to measure lymphocyte proliferation in response to antigenic stimulation (Brown, 1977; Schultz, 1982). This method allows a quantitative readout via scintillation counter by providing information about the proliferation of the cells during exposure to [^{3}H] thymidine. The ratio of radioactivity in the mitogen-treated cells compared with that in untreated cells is expressed as stimulation index.

For the LBT, 50 µl PBMC (5 x 10^6 cells / ml) in RPMI 1640 with 20 % FCS and 1 x antibiotic / antimycotic solution (Sigma, St. Louis, MO) was added in triplicate to a 96 well plate. Unstimulated control wells contained 100 µl of RPMI 1640. Stimulated sample wells contained 100 µl of Con A in RPMI with a final concentration of 6.7 µg / ml or 100 µl of the M form of PHA diluted 1:200 in RPMI 1640. Plates were incubated at 37°C in 5 % CO_2 for 48 hrs, after which the cells were pulsed by adding 100 µl [^{3}H] thymidine diluted to 10 µCi / ml in RPMI 1640 to each well. After an additional incubation for 12 - 18 hrs at 37°C in 5 % CO_2 plates were placed at -20ºC for a minimum of 30 min. Cells were harvested using a Packard Filtermate 196 (PerkinElmer, Shelton, CT) and radioactivity was measured using a Packard Topcount Microplate Scintillation Counter (PerkinElmer, Shelton, CT).

2.8. Statistical analyses

The statistical analyses were performed at the Department of Computing and Biometry, University of Wisconsin-Madison, College of Agriculture and Life Sciences using SAS 1999 - 2000 (SAS Institute Inc., Cary, NC, USA).

To standardize the data, calves were analyzed in pairs (noni fed calves – control calves). A pair consisted of two calves from the same dairy farm, based on the arrival date of the calves at the VMTH. This method of analyzing was chosen, since each pair had been exposed to the same environment over the same time frame and was fed the same pooled colostrum before they were sent to the VMTH.

a) For the bactericidal activity analysis data (n = 9 pairs) were analyzed using PROC UNIVARIATE and PROC FREQ in SAS. At each of the four time points (days 0, 3, 7, and 14) the difference between the percentage killing for each calf pair (noni puree fed - control) were computed. Analyses were done for both *E. coli* and *Staph. epidermidis* bacteria. Normal quantile plots of the differences showed that they were not normally distributed. To compare the median differences of the pairs between the two groups. Wilcoxon signed-rank tests were used. In order to determine if the differences between noni and control groups differed over time a Friedman's Test was performed. The experiment-wise error rate was controlled at the 5 % level within each bacteria type using a Bonferroni p-value correction.

b) For the LBT data (n = 9 pairs), background was removed by subtracting the unstimulated control from the mitogen stimulated value. At each of the 4 time points (days 0, 3, 7 and 14) the differences in proliferation for each calf pair (noni-fed - control) were computed for both Con A and PHA by using the background-corrected data. This difference between noni and control calves was used as the response in repeated-measures ANOVA with a first-order autoregressive correlation structure, to determine whether there was an effect of noni at each time and/or a trend in the effect of noni over the time period of the study. P-values for mean differences between noni and control at specific time points were corrected using the Bonferroni method; tests comparing the mean difference across time points were corrected using the Tukey Method. The significance level was set at $p \leq 0.05$.

c) The flow cytometry data (n = 8 pairs) was statistically analyzed in the same way as the LBT data, with separate analyses performed for Con A and PHA, for CD4 & CD25, CD8 & CD25, and TcR1-N6 & CD25 staining

combinations. PROC MIXED of SAS was used for all analyses. Significance was set at $p \leq 0.05$. Residual analysis on the above models showed that the data was approximately normally distributed. A Friedman's test (PROC FREQ of SAS) was performed to look at differences over time.

3. Results

To evaluate whether *Morinda citrifolia* fruit puree has immunomodulatory effects on the immune system of neonatal calves in the first two weeks of life T cell activation as well as lymphocyte proliferation were measured by two-color flow cytometry and lymphocyte blastogenesis test respectively. In addition to that bactericidal assay was performed to estimate the effect of noni on gram-positive and gram-negative bacterial killing.

3.1. Results of flow cytometry

The results of this experiment showed a significant increase in percent expression of CD25 on $CD4^+$ ($p = 0.0296$) and $CD8^+$ ($p = 0.0404$) T cell subsets in response to Con A in noni-fed calves on day 3 of the study compared to controls (Figure 4). A Friedman´s test did not show significant differences over time for both $CD4^+$ T cells ($p = 0.8016$) and $CD8^+$ T cells ($p = 0.0627$) in response to Con A. On day 3 of the study the highest mean difference was found followed by a gradual decrease to day 14. On day 14 levels were approximately that of day 0. No significant results in response to Con A were found for γδ TCR^+ cells. The results in response to PHA did not show significant differences in percent CD25 expression within any T cell subset.

Cell type	Day	Parametric
$CD4^+$	3	**0.0296**
$CD8^+$	3	**0.0404**
$CD4^+$	0-14	0.8016
$CD8^+$	0-14	0.0627

Table 7. Bonferroni corrected parametric p-values for the mean difference (noni – control) in CD25 percent expression on $CD4^+$ and $CD8^+$ T cells for day 3 between calf pairs (n = 8) for Con A stimulated values with unstimulated controls (background) removed.

3.1.1. CD25 expression on T cell subsets in response to Con A

The results are shown as mean difference of percent CD25 expression ± standard error mean (SEM) for parametric analysis. Due to the fact that the calves, which belong to the noni-supplemented group, have not received their first supplementation of noni fruit puree, the results of day 0 show the natural variability of CD25 expression on T cell subsets. For the purpose of comparison the results were analyzed according to the groups as they will be shown during the study.

The highest mean difference of CD25 expression in response to Con A on day 0 of the study was found on $CD4^+$ T cells (12.27 ± 11.84 %). On day 0 the mean differences of CD25 expression found on $CD8^+$ and γδ TCR^+ T cells were 7.54 ± 4.36 % and 5.24 ± 3.76 % respectively (Table 8). Although noni was not yet supplemented on day 0 T cell subsets of the calves which were selected to be treated with noni fruit puree had a naturally higher CD25 expression than the claves of the control group. No significant differences were measured at this time point. On day 3 of the study, which is approximately 4 - 5 days postpartum, flow cytometry results showed a significant increase in percent expression of CD25 on $CD4^+$ T cells in response to Con A in noni-fed calves ($p = 0.0296$). There was also a significant increase in percent expression of CD25 on $CD8^+$ T cells for noni-fed calves stimulated by the same mitogen at the same time point ($p = 0.0404$). Flow cytometry results showed an increase of mean differences of CD25 expression on $CD4^+$ and $CD8^+$ T cells by almost the same value within the first 3 days of supplementing noni. Mean differences of CD25 expression on $CD4^+$ T cells increased by 6.54 % from 12.27 ± 11.84 % to 18.81 ± 5.04 %. In the same time results of CD25 expression on $CD8^+$ T cells increased by 6.56 % from 7.54 ± 4.37 % to 14.11 ± 4.03 %. The CD25 expression on γδ TCR^+ T cells increased from 5.24 ± 3.76 % to 9.11 ± 5.80 %, but was not significant ($p = 0.6424$). Within the following days, from day 3 to day 7 of the study, all T cell subsets showed a decrease in percent expression of CD25. For $CD4^+$ T cells, which showed the highest decrease, 8.52 %, a mean difference of CD25 expression in response to Con A of 10.29 ± 11.38 % was measured on day 7. The results of CD25 expression on γδ TCR^+ T cells decreased by 5.94 %, resulting in a mean difference of 3.17 ± 3.18 %. The mean difference of CD25 expression of $CD8^+$ T cells, which was the highest on day 7 in response to Con A, decreased by only 1.24 % to 12.86 ± 6.83 % ($p = 0.4348$) The results of $CD4^+$ and γδ TCR^+ T cells decreased below the level measured on day 0 of the study. No significant differences were measured at this

time point. By the end of the study, T cell subsets $CD4^+$ and $CD8^+$, showed a decrease of CD25 expression on day 14. Mean difference of CD25 expression for $CD4^+$ T cells was measured at 6.41 ± 7.69 %. $CD8^+$ T cells of the controls had higher CD25 expression in response to Con A on the last day of the study compared to the noni-fed calves. Mean difference of CD25 expression for this T cell subset was -0.93 ± 3.21 %. Only CD25 expression on γδ TCR^+ T cells showed an increase until the end of the study (4.37 ± 6.87%). Anyhow, all mean differences of CD25 expression were below the values of day 0. No significant differences were measured at this time of the study.

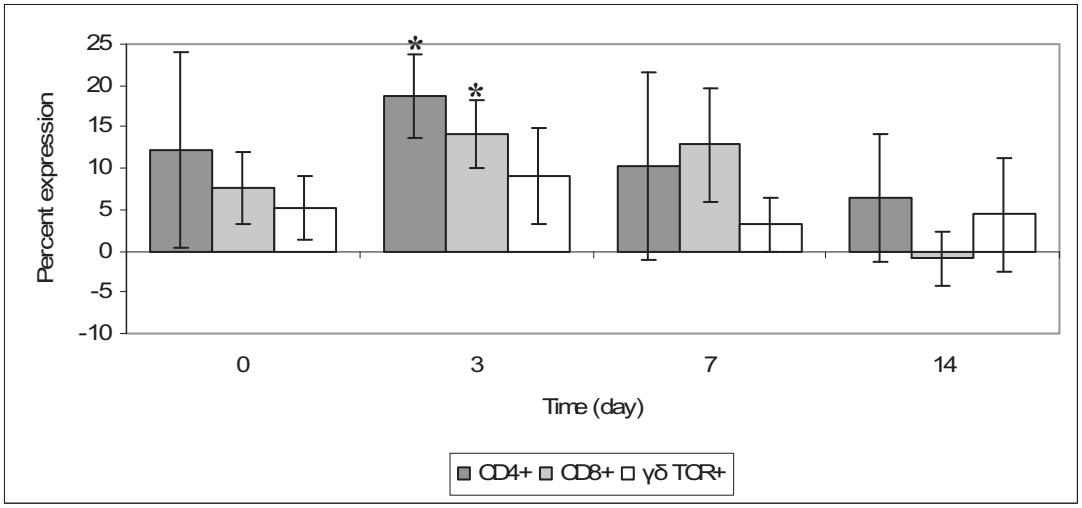

Figure 4. Mean differences (noni – control) ± SEM in CD25 percent expression on $CD4^+$ and $CD8^+$ T cells between calf pairs (n = 8) for Con A stimulated values with unstimulated controls (background) removed. The star (*) indicates a significant difference ($p \leq 0.05$).

	$CD4^+$ T cells		**$CD8^+$ T cells**		**γδ TCR^+ T cells**	
Day	MD ± SEM	P-Value	MD ± SEM	P-Value	MD ± SEM	P-Value
0	12.27 ± 11.84	1	7.54 ± 4.37	0.5108	5.24 ± 3.76	0.8268
3	**18.81 ± 5.04**	**0.0296**	**14.11 ± 4.03**	**0.0404**	9.11 ± 5.80	0.6424
7	10.29 ± 11.38	1	12.86 ± 6.83	0.4348	3.17 ± 3.18	1
14	6.41 ± 7.69	1	-0.93 ± 3.21	1	4.37 ± 6.87	1

Table 8. Mean differences percent CD25 expression ± SEM and p-values in response to Con A

A Friedman´s test, which was performed to look at differences over time, did not show significant differences for both $CD4^+$ T cells ($p = 0.8016$) and, although close to significance, $CD8^+$ T cells ($p = 0.0627$) in response to Con A.

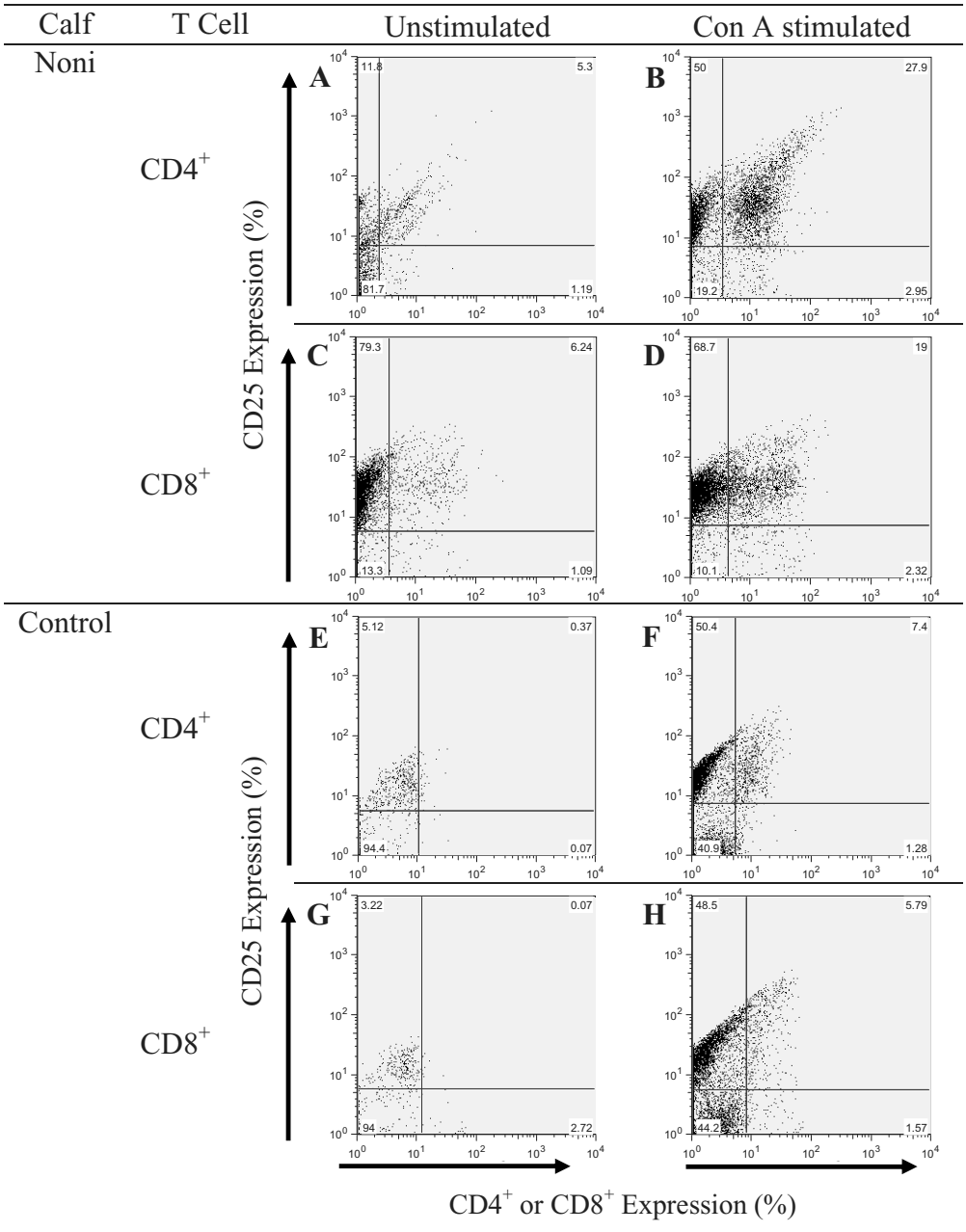

Figure 5. Two-color flow cytometry for a control and noni puree-supplemented calf on day 3 for unstimulated and Con A stimulated $CD4^+$ T cells and $CD8^+$ T cells

3.1.2. CD25 expression on T cell subsets in response to PHA

On day 0 of the study $CD8^+$ (-0.16 ± 2.25 %) and γδ TCR^+ (0.18 ± 2.98 %) T cells showed little mean differences of CD25 expression between the noni-fed calves and the controls. In contrast to the finding on the other T cell subsets, but similar to the findings in response to Con A, $CD4^+$ T cells of the noni-fed calves showed higher CD25 expression than the calves from the control group resulting in a mean difference of CD25 expression of 12.20 ± 12.30 % (Table 9). No significant differences were found at this time of the study. Although mean difference of CD25 expression of $CD4^+$ T cells increased within the first days of supplementing noni from 12.20 ± 12.30 % to 23.79 ± 11.77 %, which is the highest mean difference measured during the study, results were not significant ($p = 0.3323$). At the same time the other T cell subsets showed a decrease in mean difference of CD25 expression. $CD8^+$ T cells of the controls had higher CD25 expression than the noni-supplemented calves (-4.58 ± 4.67 %). A similar result was found on γδ TCR^+ T cells (-2.83 ± 11.91 %). In contrast to the results in response to Con A, no significant differences were found on this day of the study in response to PHA. On day 7 of the study, mean differences of CD25 expression of all three monitored T cell subsets showed different developments. Contrary to $CD4^+$ T cells, which showed a decrease of CD25 expression of noni-fed calves resulting in a mean differences of 14.16 ± 8.29 % ($p = 0.5260$), γδ TCR^+ showed an increase of CD25 expression in response to PHA of 6.82 % (4.00 ± 12.27 %). Mean differences of CD25 expression on $CD8^+$ T cells remained almost constant at -4.63 ± 7.90 %. The difference between day 3 and day 7 was only 0.05 %. No significant findings were reported for this day. Within the last week from day 7 to day 14 of the study expression of CD25 on $CD4^+$ T cells on noni-fed calves decreased further on. Mean difference of the pairs between noni-fed and control calves was -7.49 ± 13.33 %. CD25 expression on $CD8^+$ T cells increased at the end of the study. Mean difference of CD25 expression for this T cells subset on day 14 of the study was 1.75 ± 11.98 %. Results of γδ TCR^+ T cells increased from 3.4 ± 12.27 % to 4.56 ± 15.85 %. There were no significant differences found in response to PHA in any of the T cell subsets.

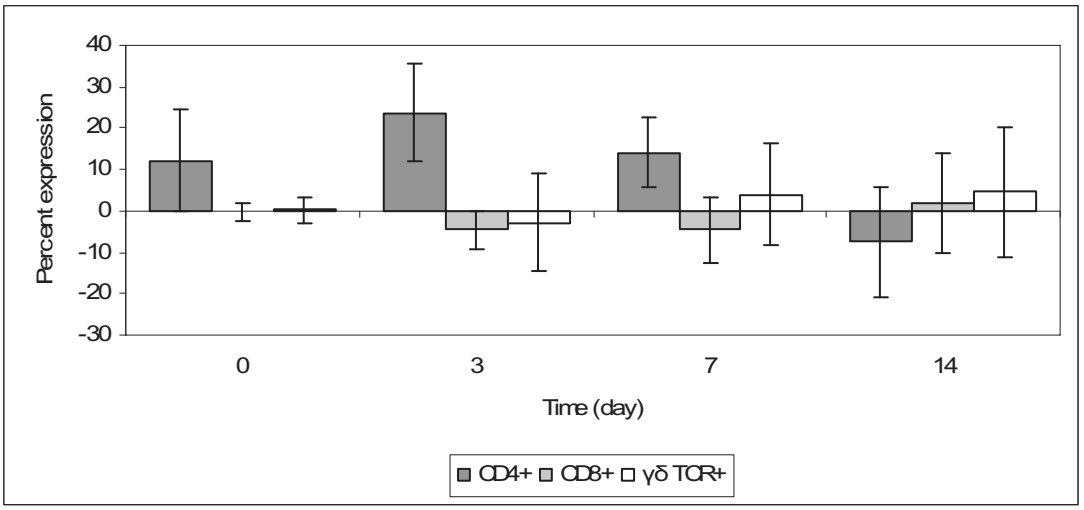

Figure 6. Mean differences (noni – control) ± SEM of CD25 percent expression on $CD4^+$, $CD8^+$ and γδ TCR^+ T cells between calf pairs (n = 8) for PHA stimulated values with unstimulated controls (background) removed.

	$CD4^+$ T cells		$CD8^+$ T cells		γδ TCR^+ T cells	
Day	**MD ± SEM**	**P-Value**	**MD ± SEM**	**P-Value**	**MD ± SEM**	**P-Value**
0	12.20 ± 12.30	1	-0.16 ± 2.25	1	0.18 ± 2.98	1
3	23.79 ± 11.77	0.3324	-4.58 ± 4.67	1	-2.83 ± 11.91	0.6424
7	14.16 ± 8.29	0.5260	-4.63 ± 7.90	1	4.00 ± 12.27	1
14	-7.49 ± 13.33	1	1.75 ± 11.98	1	4.56 ± 15.85	1

Table 9. Mean differences percent CD25 expression ± SEM and p-values in response to PHA

3.2. Results of lymphocyte blastogenesis test

The results of the lymphocyte blastogenesis test (LBT), performed in order to measure lymphocyte proliferation in response to the global mitogens Con A and PHA, shown as counts per minute (cpm), did not show significant differences between noni-fed and control calves (Table 11, 12).

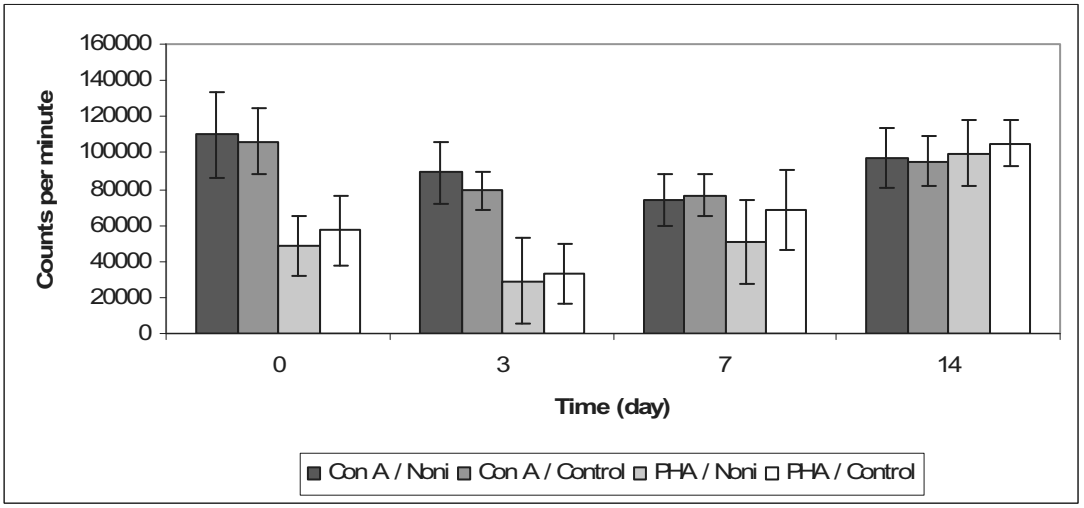

Figure 7. Counts per minute ± SEM for Con A and PHA stimulated values and unstimulated controls (background) removed.

3.2.1. Lymphocyte proliferation in response to Con A and PHA

The results of the LBT on day 0, showing the natural variability of lymphocyte proliferation, exhibited higher lymphocyte proliferation in the group which was selected to be treated with noni in response to Con A (109982 ± 23656 cpm) compared to the controls (106187 ± 16455 cpm), whereas in response to PHA the calves of the control group (57055 ± 19305 cpm) showed a higher proliferation rate compared to the noni calves (48105 ± 18039 cpm). The mean difference of lymphocyte proliferation (noni – control) before the first feeding of noni between the two groups was -8950.49 ± 27420.43 cpm in response to PHA and 3795.49 ± 25989.86 cpm in response to Con A. Within the first 3 days of the study a strong decrease of lymphocyte proliferation was measured for both groups. The results in

response to Con A of that day showed a higher lymphocyte proliferation in the calves of the noni-fed group (89010 ± 16887 cpm) compared to the controls (78918 ± 23642 cpm). The mean difference of counts per minute was 4694.14 ± 22381.36 cpm. In response to PHA the mean difference of lymphocyte proliferation of -5705.23 ± 10094.41 cpm indicates a higher proliferation rate in the control group (33363 ± 16641 cpm) when compared to the noni group (29085 ± 10544 cpm). The size of proliferation on day 3 in response to PHA was the lowest in the whole study, also when compared to proliferation in response to Con A. Whereas, the proliferation in response to Con A decreased further until day 7 of the study results in response to PHA started to increase. The proliferation in the noni-fed group increased by 21699 cpm from 29085 ± 10544 cpm to 50784 ± 11197 cpm. The control showed an increase of 35085 cpm from 33363 ± 16641 to 68448 ± 22419 cpm. By day 7 of the study the mean differences of lymphocyte proliferation in response to both Con A and PHA were -3540.65 ± 25866.28 cpm respectively -13019.30 ± 23558.81 cpm. Within the last week of the study both groups showed an increase of lymphocyte proliferation until the end of the project. The proliferation of lymphocytes in response to Con A reached almost the starting level, whereas values in response to PHA almost doubled. They increased from 48105 ± 18039 cpm in the noni calves and 57055 ± 19305 cpm in controls to 99706 ± 13688 cpm and 105144 ± 12864 cpm respectively. The median difference of proliferation between the noni-fed group and the control group in response to PHA on day 14 was – 5438.15 ± 16235 cpm. At that day the average lymphocyte proliferation level in response to PHA was for the first time higher than in response to Con A (Table 10)

	Noni Puree Fed (n = 9)		**Control** (n = 9)	
Day	**Con A**	**PHA**	**Con A**	**PHA**
0	109982 ± 23656	48105 ± 18039	106187 ± 16455	57055 ± 19305
3	89010 ± 16887	29085 ± 10544	78918 ± 23642	33363 ± 16641
7	73856 ± 14740	50784 ± 11197	76619 ± 23081	68448 ± 22419
14	96900 ± 16600	99706 ± 13688	95288 ± 18059	105144 ±12864

Table 10. There were no significant differences in lymphocyte proliferation between noni-fed and control calves at any time. Values are mean lymphocyte proliferation shown in counts per minute ± SEM.

It should be noted that the control group in response to PHA when compared with the noni-fed calves constantly showed higher lymphocyte proliferation with peak at day 7 of the study resulting in a mean difference of -13019.30 ± 23558.81 cpm. At this day lymphocyte proliferation of the control group in response to Con A was also higher than in the calves of the noni-supplemented group and thus stands in contrast to the other results in response to Con A (-3540.65 ± 25866.28 cpm).

The results of the LBT did not show any significant mean differences in lymphocyte proliferation between the noni-fed group and the control group in response to Con A or PHA (Table 12).

Mean difference lymphocyte proliferation		
Day	**Con A** (n = 9)	**PHA** (n = 9)
0	3795.49 ± 25989.86	-8950.49 ± 27420.43
3	4694.14 ± 22381.36	-5705.23 ± 10094.41
7	-3540.65 ± 25866.28	-13019.3 ± 23558.81
14	1612.09 ± 17527.06	-5438.15 ± 16235

Table 11. Mean difference of lymphocyte proliferation noni - control shown in counts per minute ± SEM

day	**0**	**3**	**7**	**14**
Con A	0.8875	0.8398	0.8956	0.9290
PHA	0.7525	0.5896	0.6005	0.7463

Table 12. P-values of LBT for Con A and PHA show no significant differences in lymphocyte proliferation between the noni-fed group and the controls

3.3 Results of bactericidal assay

The results of the bactericidal assay are shown as the median (Q_2) of the differences of pairs- noni supplemented calves – control calves in percent bacterial killing. To show the distribution of the differences the 25 % (Q_2) and 75 % quantile (Q_3) are given.

The results of the bactericidal assay showed that the median of the differences of percent *E. coli* bacterial killing increased over time (Figure 3/A). Blood taken from noni supplemented calves showed significantly enhanced bactericidal activity at day 14 compared to control calves ($p = 0.0215$) (Table 13). However, a Friedman's test showed that the size of the median of the differences of percent *E. coli* bacterial killing between the groups did not increase statistically significant ($p = 0.0534$) during the two weeks. In contrast to the above described results for gram-negative bacterial killing, no significant differences between the two groups for *Staph. epidermidis* bactericidal activity at any of the time points were measured (Table 14).

3.3.1. Bactericidal activity of whole blood against *E. coli*

On day 0 of the study, which means before the first feeding of noni, the results showed a median of the differences of 0.20 %. Although the median of the difference is marginal, the 25% and 75% quantile ($Q_1 = -18.0$; $Q_3 = 1.0$) showed, that most of the calves, which were selected for the control group had higher bactericidal killing when compared to the calves selected for the noni group. (Table 13). Until day 3 a slight increase of the median of the differences ($Q_2 = 2.80$ %) in the noni puree supplemented group was detectable ($Q_1 = -0.7$; $Q_3 = 39.0$). The upward trend, which was measured during the first 3 days of supplementing noni continued within the next days. Day 7 showed a median of the differences of bacterial killing of 18.21 % ($Q_1 = 6.21$; $Q_3 = 40.54$). Although the measured difference was not statistically significant ($p = 0.3031$). At day 14 blood obtained from noni supplemented calves showed a significantly higher bactericidal activity compared to control calves ($p = 0.0215$). The median of the differences of two groups (noni - control) of percent *E. coli* bacterial killing increased by the end of the study up to 23.70 % ($Q_1 = 5.4$; $Q_3 = 40.9$). A Friedman´s test, which was

performed to measure the development over time, showed, that the overtime increased median difference did not increase statistically significant (Friedman's Test, p = 0.0534).

E. coli				
Day	**Median of the differences %**	**Quantile 25 %**	**Quantile 75 %**	**P-Value**
0	0.20	-18.00	1.00	0.7013
3	2.80	-0.70	39.00	0.2715
7	18.21	6.21	40.54	0.3031
14	23.70	5.40	40.90	0.0215

Table 13. Median of the differences of pairs (noni – control) in percent *E. coli* bactericidal killing and p-values.

3.3.2. Bactericidal activity of whole blood against *Staph. epidermidis*

At day 0 of the study a median of the differences of *Staph. epidermidis* bacterial killing of 0.69 % was measured (Q_1 = 0.00; Q_3 = 7.60) (Table 8). This result exhibits a lower natural variability when compared to bacterial killing of *E. coli.* Within in the first 3 days of feeding noni the median of the differences in bactericidal killing increased to 4.06 % (Q_1 = -18.01; Q_3 = 20.21). In contrast to the phagocytic activity of whole blood in response to *E. coli* no further increase of the added benefit of noni over time was detectable between day 3 and day 7. At this point the control group showed a higher phagocytic activity, resulting in a median of the differences of -14.42 % (Q_1 = -20.91; Q_3 = 1.46). Although still showing a higher *Staph. epidermidis* bacterial killing the median of the differences of the pairs decreased until day 14, -2.7 % (Q_1 = -7.73; Q_3 = 3.11). There were no significant differences between the groups for *Staph. epidermidis* bactericidal activity at any of the time points.

Staph. epidermidis				
Day	**Median of the differences %**	**Quantile 25 %**	**Quantile 75 %**	**P-Value**
0	0.69	0.00	7.60	1
3	4.06	-18.01	20.21	1
7	-14.42	-20.91	1.46	1
14	-2.70	-7.73	3.11	1

Table 14. Median of the differences of pairs (noni – control) in percent Staph. epidermidis bactericidal killing and p-values.

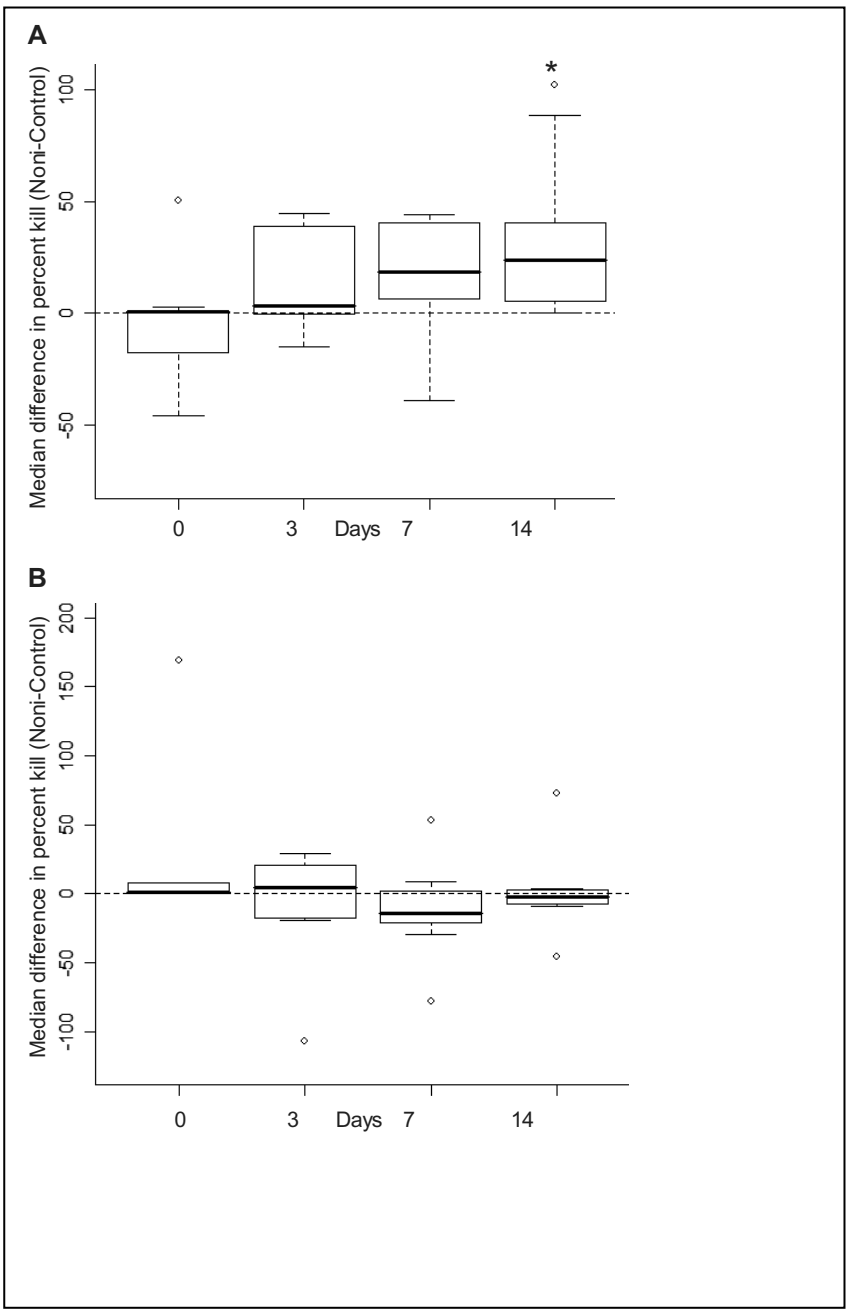

Figure 8. Median of the differences of calf pairs (noni - control; n = 9) (**A**) percent killing of *E. coli* (**B**) *Staph. epidermidis.* The dark line indicates the median (Q_2); the box contains the middle 50 % of the data points; the whiskers extend out to include any point within 150 % of the interquartile range (IQR); a circle indicates a point outside of 1.5 x IQR. The star (*) indicates significance ($p < 0.05$).

4. Discussion of the results

In the present study we examined whether *Morinda citrifolia* fruit puree, a phytochemical based immunomodulator, fed in conjunction with milk replacer could affect T cell activation and proliferation as well as bactericidal activity of whole blood in neonatal calves within the first 2 weeks of life. To evaluate the effects of the noni puree on the immunity of newborn calves, T cell activation, more precisely CD25 expression, and proliferation were measured by two-color flow cytometry and by LBT respectively in response to the global T cell mitogens Con A and PHA. To quantify bactericidal activity of whole blood a bactericidal assay was performed.

4.1. Effects of *Morinda citrifolia* fruit puree on the expression of CD25 on T cell subsets $CD4^+$, $CD8^+$ and γδ TCR^+

Results from flow cytometry showed that immunoglobin competent calves receiving milk replacer blend with noni fruit puree had a significant increase in CD25 expression on $CD4^+$ and $CD8^+$ T cells on day 3 of the study, which approximately is day 4 - 5 postpartum, in response to Con A when parametric analysis was performed. Starting on day 3 of the study, showing the highest mean difference, a gradual decrease to day 14, where levels were approximately that of day 0, was detectable**.**

As described above (chapter 1.1.2.1.) effective cell-mediated immune responses depend on a full repertoire of functional T cells, including $CD4^+$ T helper cells, cytotoxic $CD8^+$ and γδ TCR^+ T cell subsets. $CD4^+$ T helper cells play an important role in promoting cellular immune responses such as an early Th1 cell-mediated inflammatory response, involving macrophage activation, and coordinating the overall adaptive immune response through secretion of cytokines, such as IL-2 and IFN-γ. These specific cytokines for example play a major role in the defense against the multiplicity of infections via boosting cellular immune responses or delayed hypersensitivity reactions (Boehm et al., 1997; Pieters, 2000). $CD8^+$ cytotoxic T cells are also crucial for an effective immune response. Their major task is to make invaders become susceptible to humoral immunity by attacking and lysing infected cells (Barry and Bleackley, 2002). *γδ* TCR^+ T cell subsets make up

the largest fraction in the blood of neonatal calves. Amongst multiple other effects such as cytokine production, cytotoxic activity (Welsh et al., 1997; Boom, 1999; Hensmann and Kwitakowski, 2001; Passmore et al., 2001) and immunomodulation (Kaufmann et al., 1993; Mombaerts et al., 1993; D´Souza et al., 1997; Smith et al., 1999) are discussed in the literature.

Calves are born agammaglobulinemic and thus uptake of immunoglobulins via colostrum, which contains besides immunoglobulins, large quantities of leukocytes and cytokines, such as IFN-γ, IL-1β, IL-4, IL-6, IL-18 and TNF-α (Hagiwara et al., 2000; Muneta et al., 2005), is necessary for an effective immune response during the first days of life. The highest concentration of cytokines in the colostrum, which are considered to contribute to the maturation of the neonatal immune system (Hagiwara et al., 2000) can be measured immediately after parturition. Hagiwara et al. (2001) reported an immune stimulatory effect of IL-1β by activation of circulating lymphocytes. In 2003 Yamanaka et al. showed that colostral cytokines, such as IL-1β, TNF-α and IFN-γ, contribute to the enhancement of neonate immunity through up regulation of IL-2 mRNA production and increased expression of the mature IL-2 receptor CD25. A study by Jensen and Schultz (1990) revealed that bovine peripheral blood mononuclear leukocytes incubated with IL-2, IL-4, TNF-α, and IFN-γ significantly enhanced natural cell-mediated cytotoxicity against K562 human myoblastic and Yac-1 mouse lymphoma cell lines. Colostral cells and cytokines, major components of colostrum, reportedly enter neonatal circulation quickly after ingestion with a peak 12 - 24 hrs later (Hagiwara et al., 2001; Liebler-Tenorio et al., 2002; Yamanaka et al., 2003b). This time point is approximately day 0 of the study and of gut closure, which occurs around 1 - 2 days of age (Bush and Staley, 1980). The significant findings made on day 3 of the study lead to the assumption that noni might have an effect on absorbed maternal cells as well as on cytokines, since the activation of $CD4^+$ and $CD8^+$ T cells posses similarities to the trafficking pattern and concentration of cells and cytokines found in colostrum, which can be measured in the circulation of neonates after the ingestion of colostrum. This hypothesis is supported by the fact that the noni puree was administered in milk replacer, completely devoid of colostral cells.

The findings of this study are in line with the observation by Hirazumi and Furusawa (1999) indicating that noni is capable of stimulating the release of IFN-γ, IL-1β, IL-10, IL-12 p70, TNF-α and NO and at the same time suppressing IL-4 production from murine effector cells. However, an explanation for the activation

mechanisms was not given by this study. A possible way would be the activation through APCs such as macrophages and DCs, which recognize PAMPs by their TLRs. This would lead to an up-regulation of cell-surface expression of co-stimulatory and MHC class II molecules. In murine models it was shown, that bacterial infection induces Th1-cytokine production such as IL-12, which induces NK cells and naïve T cells to differentiate into Th1 cells (Werling and Jungi, 2003). These cells produce the cytokines IFN-γ and IL-2, which are known to activate more Th1 cells, $CD8^+$ CTL, NK cells, and INF-γ and IL-5 production. Thus by enhancing the activity of NK cells, noni-ppt is a potent stimulator of immunity. Price et al. (2006) showed WC 1^+ γδ T-cells, which are significantly more abundant in the peripheral blood of neonatal calves compared to adult animals, were stimulated by IL-12 and IL-18 to secrete large quantities of IFN-γ. Additionally, Hope et al. (2002) demonstrated that *Mycobacterium bovis* bacillus Calmette-Guerin-infected dendritic cells stimulated $CD3^-$ $CD8^+$ NK-like and $CD3^+$ $CD8^+$ T cell proliferation. The NK-like cells were the major population of IFN-γ producers and may play a role in Th1-biased immune responses. If indeed Noni does up-regulate TNF-α, and IFN-γ there may be a direct increase in natural cell-mediated cytotoxicity through the significantly enhanced activation of $CD8^+$ T cells.

In a recent study on the mechanisms which are involved in the immunomodulatory and anti-inflammatory effects of *Morinda citrifolia* by Palu et al. (2008) another way of action, supporting the above described findings, was discovered. Palu et al. reported a decreased IL-4, but increased IFN-γ production after oral administration of Tahitian Noni Juice™ *ad libitum* for 16 days in C57B/6J mice. This effect potentially was mediated by activation of the cannabinoid 2 (CB_2) receptor, whereas the cannabinoid 1 (CB_1) receptor was inhibited. The CB_2 receptor plays an important role in immunomodulation and anti-inflammation (Massa et al., 2004) and can be found especially on hemopoietic cells which differentiate to myeloid and lymphoid cells (Munro et al., 1993). Gertsch et al. (2004) reported an immune stimulating effect of *Echinacea* by modulating the TNF-α mRNA expression in human monocytes / macrophages via the CB_2 receptor. It is conceivable that the significant increase in $CD8^+$ T cell activation and CD25 expression may be due to the stimulating effects of noni on the production of important cytokines such as TNF-α, IL-1β and IFN-γ (Hirazumi and Furusawa, 1999). These findings and the results of this study suggest that the CTL associated,

Th1 cell-mediated, immune response may be due to direct or indirect stimulation by the noni fruit puree on IFN-γ production.

4.2. Effect of *Morinda citrifolia* fruit puree on lymphocyte proliferation in response to Con A and PHA

As described above in chapter 4.1. $CD4^+$ and $CD8^+$ T cells from noni fed calves compared to control calves showed a significant higher expression of CD25 on day 3 of the study. In combination with a significant effect over time of $CD8^+$ T cells activation of T cells in the noni group these results implicate an increase in T cell proliferation in the noni group, since the expression of CD25 which is the α-chain of the interleukin receptor increases the affinity to bind IL-2. IL-2 is essential for inducing clonal T cell proliferation. Contrary to expectations, the results of the lymphocyte blastogenesis test failed to show significant differences in proliferation in response to the global T cell mitogens Con A or PHA between the two groups, the noni puree fed group and the control group.

Franklin et al. (1994) found that proliferation of bovine mononuclear leukocytes stimulated by pokeweed mitogen remained relatively constant day 6 through 14 and peaked 12 d after seeding, while unstimulated control cells decreased over time with the lowest levels (<20%) 12 d after seeding. In this study, according to the used protocol (Schultz, 1982), cells were counted approximately 3 days after seeding, which may not have allowed adequate incubation time to see a significant effect as lymphocyte clonal expansion peaks around 6 - 7 days (DeFranco et al., 2007). No time course was executed, thus no information on possible trends in proliferation was given. Furthermore, the samples had not been analyzed for lymphocyte composition; therefore it is difficult to determine if one cell subset was affected differently than another. Perhaps the lack of proliferation of one cell subset may have neutralized an increased response in another. A more appropriate test would have entailed fluorescent antibody labeling of each cell subset, staining cells with Paul Karl Horan (PKH) or carboxyfluorescein succinimidyl ester (CFSE) dye and analyzing proliferation via flow cytometry (Givan et al., 1999; Sandbulte and Roth, 2004). Furthermore, Clover and Zarkower (1980) found colostrum-fed calves had significantly higher proliferation counts than colostrum-deprived calves. As all calves in the study received adequate levels of colostrum, LBT results may have

been altered by this effect, explaining why no significant difference was observed between control and noni-fed calves. In consideration of these facts tests with colostrum-deprived calves have to be done.

4.3. Effect of *Morinda citrifolia* fruit puree on the bactericidal activity of whole blood against *E. coli* and *Staph. epidermidis*

The results of the phagocytic assay showed that calves receiving noni had increasing phagocytic activity against gram-negative bacteria, while phagocytosis of gram-positive bacteria seems to be unaffected resulting in a significantly better gram-negative bacterial killing than controls on day 14 of the study. This finding suggests that noni puree may enhance bactericidal activity against gram-negative bacteria in the neonatal calf.

In 2005 *Morinda citrifolia* was tested by Zaidan et al. amongst other medical plants for their antimicrobial activity by using disc diffusion method. A methanol leaf extract from *Morinda citrifolia* showed potential antibacterial activity to gram-positive *S. aureus* ATCC 25923 at the highest used concentration of 1000 µg / disc. No antibacterial activity against the other tested bacteria including *Methicillin* resistant *Staphylococcus aureus* (MRSA), *Klebsiella pneumoniae* (IMR K25/96), *Pseudomonas aeruginosa* ATCC 27853, and *Escherichia coli* ATCC 25922 was reported. Analyses of the active antimicrobial agents were not performed in this study. Due to the high concentration used and no effect on the other tested bacteria the antibacterial activity of Noni fruit puree used in this study can be neglected.

Bactericidal activity directed against *E. coli* could proceed by three different pathways. Pathogen recognition receptors recognize specific patterns of the LPS component of the outer membrane. *E. coli* bacteria undergo direct phagocytosis by neutrophils or T helper cells recognize *E. coli* antigens, which are expressed on major histocompatibility complex (MHC) class II molecules on antigen presenting cells such as macrophages and B cells. In consideration of the limited scope of this study a definitive explanation can not be postulated. Nevertheless, some plausible mechanisms are worth noting. Colostral antibodies to lipopolysaccharides (LPS), which can be acquired by either natural exposure or vaccinial stimulation of the dam, can have a positive interaction with components of the noni fruit puree. Since calves were obtained from farms with gram-negative core mutant vaccine protocols

in dry cows and heifers, which is part of mastitis control program, presumably passively acquired anti-LPS antibodies were present to some titer level in all calves. It is also unknown if antibodies to passively acquired antibody to gram-positive organisms like *Staph. epidermidis*. were present. Furthermore noni puree may non-specifically stimulate innate immunity of the calf resulting in enhanced phagocytic activity, or it may potentiate immune functions by enhancing colostral acquired immunity.

4.3.1. Enhancement of bactericidal activity by stimulation of innate immunity

Stimulation of the innate immune response in neonatal calves through noni puree might be induced by non-specific stimulation of phagocytes, in particular of macrophages. Immune cells might be activated through binding noni extract components to Toll-like receptor 4 (TLR4), CD14, complement receptor 3 (CR3), scavenger receptor, dectin-1 and mannose receptor. These are the same receptors involved in the binding of bacterial polysaccharides. Activated cells respond with increased reactive oxygen species (ROS) and nitric oxide (NO) secretion as well as with enhanced production of pro-inflammatory cytokines such as TNF-α, IL-1, IL-6, IL-8, IL-12 and IFN-γ. Botanical arabinogalactans-containing polysaccharides, which also can be found in the noni-ppt (Bui et al., 2006) extracted from *Juniperus scopolorum*, are demonstrated to cause the described effects in human and murine macrophages (Schepetkin et al., 2005). In addition, extracts obtained from *Silene vulgaris*, also containing arabinogalactans, enhanced phagocytosis and lysozymal activity of rat and human macrophages (Popov et al., 1999). In consideration of the fact arabinogalactans being also part of the cell wall in Corynebacteria, Mycobacteria and Nocarida (Daffe et al., 1993) it can be assumed, that the response to botanical arabinogalactans partially mimics the response to bacterial arabinogalactans and is thus a stimulator of innate immunity.

4.3.2. Enhancement of bactericidal activity by stimulation of colostral leukocytes

Another possible mechanism of enhancing bactericidal activity of whole blood might be mediated by colostral leukocytes. Riedel-Caspari and Schmidt (1991) investigated the influence of colostral leukocytes on the immune system of the neonatal calf with emphasis, inter alia, on the effects on lysozyme activity and bactericidal activity against *E. coli*. Menge et al. (1998) reported that neutrophils from neonatal calves have decreased phagocytic capacity against *E. coli* when compared to older calves. They discovered a several weeks lasting stimulatory effect on lysozyme activity in sera of calves fed with complete colostrum compared to calves fed cell-depleted colostrum as well as milk substitutes. Activity of lysozyme reached peak activity on day 14 post partum. Riedel-Caspari and Schmidt (1991) assumed a colostral T cell-mediated stimulation of lysozyme production in phagocytes, in particular of macrophages. In addition, Riedel-Caspari and Schmidt (1991) reported an increase of bactericidal activity of whole blood against a porcine haemolytic *E. coli* strain after colostrum feeding. Two groups obtaining milk substitutes instead of colostrum showed a significantly decreased bactericidal index compared to the colostrum fed groups. Within the colostrum fed group calves receiving complete colostrum showed a significant higher increase of the reduction of colony forming units (CFU) than animals fed with cell-depleted colostrum at day 2 and 3 of the study. These results indicate a stimulatory effect of colostral leukocytes within their colostral environment on bactericidal activity of whole blood against *E. coli*. In 1993 Riedel-Caspari confirmed the results of his previous study, but the mechanism of colostral leukocytes influencing bactericidal activity remains unclear. A direct antimicrobial effect of colostral leukocytes is discussed as well as stimulation of the immune response by colostral effector cells. Riedel-Caspari demonstrated that leukocytes from mastitic glands increased elimination by about day 14 post partum. Inflammatory cytokines and antigenic contact may have stimulated effector cells which comprise T helper cells, cytotoxic T cells (CTL) and natural killer cells (NK).

Menge et al. (1998) reported an increase of phagocytic activity of polymorphonuclear leukocytes (PMNL) in neonatal calves after colostrum ingestion. Sugisawa (2001) supported these results by reporting bovine colostrum enhances phagocytic activity in vitro. Ultrafiltered bovine whey product was also shown to enhance neutrophil function (Roth et al., 2001). Coincidently, noni was

reported to enhance production of several pro-inflammatory cytokines by murine effector cells, such as priming cytokines (IL-1, IL-6 and TNF-α) found in bovine colostrum (Hagiwara et al., 2000). Yamanaka et al. (2003) suggested colostral cytokines such as IL-1β, TNF-α, IFN-γ play a role in enhancing neonate immunity through up regulation of IL-2 production and mature IL-2 receptors (CD25) of peripheral blood mononuclear cells (PBMC). The role of colostral leukocytes and cytokines in enhancing neonatal immunity has recently been an area of active research (Kenzel and Henneke, 2006). Previous to these findings Hagiwara et al. (2000) reported that pretreatment of PBMC from newborn calves with IL-1β significantly enhanced CD25 expression and promoted a mitogenic response to Con A. These cytokines contribute to the stimulation of effector cells including CTL and NK cells. Activated cells kill bacteria and produce INF-γ which stimulates macrophages. This hypothesis is paralleled with our previous described findings showing that noni puree was capable of significantly enhancing the expression of the activation marker CD25 on $CD8^+$ T cells.

Given the recent advances in phytochemical immunopharmacology and most specifically the role of polysaccharides as immunomodulators, it is tempting to speculate that noni puree may stimulate the innate immune response by modulating colostral and/or neonatal mononuclear cells (Bui et al., 2006; Schepetkin and Quinn, 2006).

5. Conclusion

The results of this study show that *Morinda citrifolia* fruit puree has a significant stimulatory effect on the activation of $CD4^+$ and $CD8^+$ T cells on day 3 after supplementing noni fruit puree. In addition to these findings made by flow cytometry a significant stimulatory effect on bactericidal activity against *E. coli* of neonatal calves on day 14 of the study was measured by a bactericidal assay. No effect on lymphocyte proliferation was detected in LBT.

It is conceivable that the significant increase in CD25 expression on $CD4^+$ and $CD8^+$ T cells may be due to the stimulating effects of noni on the cytokines TNF-α, IL-1β and IFN-γ found in ingested colostrum (Hirazumi and Furusawa, 1999). However, it is not possible to give a specific explanation of the mechanism which evoked this significant activation of T cells after noni fruit puree ingestion. Both a direct influence on T cells via endogenous cytokines as well as indirect stimulation through other cellular immune components might be possible. It will be crucial to determine if noni indeed has a stimulatory effect on colostral leukocytes as assumed. Further trials with colostrum fed and colostrum deprived calves could give answers. Additional studies are required to verify the influence of noni on cytokine stimulation and up-regulation in bovine colostrum as well as in the neonatal calf. A polymerase chain reaction (PCR) study to measure cytokine messenger RNA (mRNA) production as well as an enzyme linked immunoabsorbent assay (ELISA) measuring produced cytokine would be appropriate and potentially could provide a link between the reported *in vitro* enhancement of the immune system and the assumed mechanisms.

The results of the bactericidal assay indicate that noni puree supplementation enhances the innate immune response in neonatal calves resulting in a significantly diminished *E. coli* growth. On the other hand no impact on *Staph. epidermidis* bactericidal activity was detectable. These findings may be of great importance for the bovine neonate. Diseases associated with failure of passive transfer, such as gram-negative septicemia or gram-negative enteric diseases, including *Colibacillosis* and *Salmonellosis*, play a major role in causing morbidity and mortality in neonatal calves (Rebhun, 1995; Vaala and House, 2002). The clinical relevance, improved survival and reduced morbidity, of the reported effect depends on the time when the enhanced killing of gram-negative bacteria appears and will have the ability to prevent clinical cases, since septicaemia associated with *E. coli* infection as well as specific forms of enteric *E. coli* infection start to occur during

the first 7 days of life. Although there was an upward trend for *E. coli* killing during the study starting early a significant difference between the two groups was only found on day 14. Although gram-negative infections play a superior role as widespread diseases of newborn calves within in the first weeks of life, infections caused by gram-positive bacteria, such as umbilical infections, are also of importance. The absence of *in vitro* bactericidal effect against gram-positive species may have consequences for general neonatal health. Further *ex vivo* studies are needed to explain the contrary results of the inhibition of gram-negative growth and bactericidal effect on gram-positive bacteria. In addition to that the precise mechanism of gram-negative inhibition might be found and explained. Besides additional *in vitro* studies the clinical application of noni puree and its effect in promoting calf health and production have to be tested in larger trials, for example on commercial calf raising farms. The potential of *Morinda citrifolia* fruit puree in decreasing morbidity and mortality in a gram-negative endotoxin or live pathogen challenge model could be tested that way.

Based on the findings made in this study *Morinda citrifolia* fruit puree has the potential to reduce morbidity and mortality in newborn calves by increasing CD25 expression on $CD4^+$ and $CD8^+$ T cell subsets as well as via enhancing the bactericidal activity against the gram-negative bacteria *E. coli*. However, further studies will have to be performed to clarify and to specify the cellular mechanisms responsible for the immune modulating effects of *Morinda citrifolia* on assumably colostral immunoglobulins, leukocytes and cytokines, which were absorbed by the neonatal calf.

6. Annex

6.1. List of abbreviations

ACD-A	Acid Citrate Dextrose-A
ANOVA	Analysis of variance
AP-1	Activation protein-1
APC	Antigen presenting cell
APT	Adequate passive transfer
BCR	B cell receptor
BHV-1	Bovine herpesvirus-1
BRM	Biologic response modifier
BRSV	Bovine respiratory syncytial virus
BVDV	Bovine viral diarrhea virus
Ca^{++}	Calcium
CD	Cluster of differentiation
CD2	Cluster of differentiation 2
CD4	Cluster of differentiation 4
CD3	Cluster of differentiation 3
CD8	Cluster of differentiation 8
CD14	Cluster of differentiation 14
CD25	Cluster of differentiation 25
CD40L	Cluster of differentiation 40 ligand
CFSE	Carboxyfluoresceinsuccinimidylester
cfu	Colony forming units
cm	Centimeter
Con A	Concanavalin A
COX-1	Cyclooxigenase-1
COX-2	Cyclooxigenase-2
cpm	Counts per minute
CR3	Complement receptor 3
CTL	Cytotoxic T lymphocyte

dl	Deciliter
DMBA	Dimethylbenzo(α)anthracene
D-PBS	Dulbecco´s phosphate buffered saline
EU	European Union
FCS	Fetal calf serum
FDA	Food and Drug Administration
FPT	Failure of passive transfer
g	Gram
g	G-force
gal	Gallon
hr	Hour
hrs	Hours
IACUC	Institutional Animal Care and Use Committee
IFN	Interferon
IFN-γ	Interferon-γ
IFR3	Interferon regulatory factor 3
IgA	Immunoglobulin A
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL-1	Interleukin-1
IL-1β	Interleukin-1β
IL-1ra	Interleukin-1 receptor antagonist
IL-2	Interleukin-2
IL-4	Interleukin-4
IL-5	Interleukin-5
IL-6	Interleukin-6
IL-12	Interleukin-12
IL-13	Interleukin-13
IL-18	Interleukin-18

l	Liter
LBP	LPS-binding protein
lbs	Pounds
LBT	Lymphocyte Blastogenesis Test
LLC	Lewis lung carcinoma
LPS	Lipopolysaccharide
m	Meter
MCM	Modified Chee´s Medium
mg	Milligramm
Mg^{++}	Magnesium
µl	Mikroliter
min	Minute
minm	Minimum
ml	Milliliter
mm	Millimeter
mM	Millimolar
MHC class I	Major histocompatibility complex class I
MHC class II	Major histocompatibility complex class II
MRSA	Methicillin resistant *Staphylococcus aureus*
MSM	Methylsulfonylmethane
NF-κB	Nuclear factor-kappa B
NK cells	Natural killer cells
NO	Nitric oxide
NOD	Nucleotide-binding oligomerization domain
Noni-ppt	Noni-precipitant
oz	Ounce
p.	Page
PAF	Platelet activation factor
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PCV	Packed cell volume

PHA	Phytohemagglutinin
PKH	Paul Karl Horan
PRR	Pattern recognition receptor
qts	Quarts
q.s.	Quantum satis
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPMI 1640	Roswell Park Memorial Institute 1640
SD	Sprague-Dawley
sec	Second
SR	Scavenger receptor
TCR	T cell receptor
Th	T helper cell
Th1	T helper 1 cell
Th2	T helper 2 cell
TLR	Toll-like receptor
TLR4	Toll-like receptor 4
TNF-α	Tumor necrosis factor-α
TNJ	Tahitian Noni Juice
TP	Total protein
TSB	Tryptic soy broth
UVB	Ultraviolet B
var	Variety
WC1	Workshop Cluster 1
WHO	World Health Organization

6.2. Data

6.2.1 Flow cytometry

Values presented in Tables 15 – 18 were acquired by two-color flow cytometry. Results are shown in percent expression of CD25 on T cell subsets $CD4^+$, $CD8^+$ and $\delta\gamma$ TCR^+ in response to the global mitogens Con A and PHA**.**

	Con A (n = 8)			**PHA** (n = 8)		
Pair	**$CD4^+$**	**$CD8^+$**	**γδ TCR^+**	**CD4**	**CD8**	**γδ TCR^+**
1	-27.20	30.30	0.44	-13.79	0.03	0.17
2	3.00	9.10	9.12	13.80	11.11	15.69
3	10.85	-5.73	-1.28	29.21	-0.03	0.89
4	29.36	18.15	28.15	-0.49	-0.18	1.57
5	-4.39	7.77	4.81	-12.03	-0.88	3.12
6	15.03	8.02	-1.62	13.14	3.87	-0.17
7	83.33	-5.65	-6.17	87.83	-11.38	-14.38
8	-11.85	-1.62	8.45	-20.05	-3.80	-5.53

Table 15. Percent expression of CD25 noni - control on T cells on day 0

	Con A (n = 8)			**PHA** (n = 8)		
Pair	**CD4**	**CD8**	**γδ TCR^+**	**CD4**	**CD8**	**γδ TCR^+**
1	33.10	31.90	14.35	-0.60	7.74	55.15
2	11.87	15.11	19.42	64.37	-31.79	-11.18
3	17.80	13.40	-12.43	-12.03	-13.83	-67.46
4	46.90	16.90	39.5	-0.90	-2.10	4.4
5	16.30	23.75	3.04	63.10	9.97	2.31
6	3.61	-3.02	-0.91	62.66	-3.55	8.38
7	9.06	0.00	-5.56	7.01	0.09	-0.48
8	11.82	14.80	14.96	6.70	-3.18	-13.74

Table 16. Percent expression of CD25 noni - control on T cells on day 3

	Con A (n = 8)			PHA (n = 8)		
Pair	**CD4**	**CD8**	**γδ TCR$^+$**	**CD4**	**CD8**	**γδ TCR$^+$**
1	-5.90	38.10	18.6	10.30	0.00	0
2	41.90	38.90	1.04	-14.79	-38.57	-54.48
3	72.30	2.47	2.82	67.60	-4.63	33.42
4	6.92	9.50	13	7.99	2.81	6.16
5	9.30	-1.20	-1.39	12.28	-34.94	-32.83
6	-24.20	6.21	3.13	9.00	1.39	3.24
7	0.90	/	-11.28	15.10	29.61	52.82
8	-18.92	-3.95	-0.18	5.77	7.32	23.63

Table 17. Percent expression of CD25 noni - control on T cells on day 7

	Con A (n = 8)			PHA (n = 8)		
Pair	**CD4**	**CD8**	**γδ TCR$^+$**	**CD4**	**CD8**	**γδ TCR$^+$**
1	30.05	3.37	2.73	18.15	26.67	19.43
2	-13.40	4.75	8.21	-33.80	-13.65	-16.29
3	-18.80	0.09	4.9	-11.73	5.13	5.45
4	39.20	13.68	6.73	46.33	55.89	80.64
5	-14.67	-2.81	-27.3	-21.27	-46.01	-76.6
6	10.00	-16.08	43.52	38.61	8.25	31.62
7	0.20	-0.70	2.64	-42.00	-39.20	-12.76
8	18.69	-9.70	-3.5	-54.21	16.90	5

Table 18. Percent expression of CD25 noni - control on T cells on day 14

6.2.2. Lymphocyte blastogenesis test

The results of the LBT shown in Tables 19 – 26 are presented as counts per minute.

6.2.2.1. Lymphocyte proliferation in response to Con A

Pair	Noni cpm	Control cpm	Difference cpm
1	77966.77	185045.4	-107078.63
2	144182	161730.9	-17548.83
3	267837.3	89919.63	177917.63
4	13986.03	25707.93	-11721.90
5	120683.4	79019.33	41664.03
6	72248.7	66571.63	5677.07
7	64374.8	103183.2	-38808.37
8	126673.5	104673.3	22000.27
9	101887	139828.8	-37941.83

Table 19. Counts per minute in response to Con A on day 0

Pair	Noni cpm	Conrol cpm	Difference cpm
1	156925.4	19931.1	136994.30
2	92191.8	55933.43	36258.37
3	19613.53	7498.767	12114.77
4	132196.3	/	132196.33
5	143318.1	216767.8	-73449.77
6	79672.57	90324.27	-10651.70
7	86869.87	135647.3	-48777.47
8	79362.23	87060	-7697.77
9	10944.03	18181.67	-7237.63

Table 20. Counts per minute in response to Con A on day 3

Pair	Noni cpm	Control cpm	Difference cpm
1	86691.03	29953.43	86691.03
2	102626.6	24369.17	78257.47
3	30940.57	2423.133	28517.43
4	151239.6	158919.6	-7679.97
5	65979.07	185167.1	-119188.07
6	48353.83	48406.6	-52.77
7	23174.1	84550.37	-61376.27
8	/	79158.6	-79158.60
9	81845.23	/	81845.23

Table 21. Counts per minute in response to Con A on day 7

6.2.2.2. Lymphocyte proliferation in response to PHA

Pair	Noni cpm	Control cpm	Difference cpm
1	9511.4	46827.32	-37315.92
2	60617.83	19075.8	41542.03
3	136804.1	163605.2	-26801.03
4	96824.2	123274.4	-26450.17
5	73791.57	106959.7	-33168.13
6	81756.23	131264.3	-49508.10
7	97430.95	23386.13	74044.82
8	181569.4	90861.7	90707.70
9	133798	152340.3	-18542.37

Table 22. Counts per minute in response to Con A on day 14

Pair	Noni cpm	Control cpm	Difference cpm
1	4487.833	58213.23	-53725.40
2	8892.933	3405.467	5487.47
3	123743.6	26299.97	97443.63
4	633.7167	10078.83	-9445.12
5	24506.7	24526.8	-20.10
6	111595.3	3006.267	108589.07
7	8995.767	156310.5	-147314.73
8	122185.9	110361.7	11824.13
9	27903.07	121296.5	-93393.40

Table 23. Counts per minute in response to PHA on day 0

Pair	Noni cpm	Control cpm	Difference cpm
1	86021.7	83342.97	2678.73
2	43923.8	2103.233	41820.57
3	28730.73	44451.47	-15720.73
4	40502.8	/	40502.80
5	61810.4	124087.8	-62277.43
6	163.8667	1606.667	-1442.80
7	-1137.47	1411.267	-2548.73
8	1304.8	739.6667	565.13
9	443.2667	9159.833	-8716.57

Table 24. Counts per minute in response to PHA on day 3

Pair	Noni cpm	Control cpm	Difference cpm
1	79709.37	58114.37	21595.00
2	3736.267	-336.867	4073.13
3	13833.67	25577.67	-11744.00
4	89489.17	167481.9	-77992.77
5	69162.4	49467.67	19694.73
6	67947.53	1016.667	66930.87
7	29126.6	142818.7	113692.13
8	/	103446.6	103446.57
9	53265.35	/	53265.35

Table 25. Counts per minute in response to PHA on day 7

Pair	Noni cpm	Control cpm	Difference cpm
1	21075.97	113085.8	-92009.78
2	119994.3	83501.27	36493.07
3	163621.5	192826.2	-29204.70
4	102925.8	126883.7	-23957.87
5	101407.5	99994.57	1412.97
6	89740.37	85952.5	3787.87
7	101039.7	69240.63	31799.02
8	135477.1	66085.5	69391.57
9	62071.33	108726.8	-46655.47

Table 26. Counts per minute in response to PHA on day 14

6.2.3. Bactericidal assay

The results of the bactericidal assay presented in Tables 27 – 34 are shown as percent bacterial killing of *E. coli* and *Staph. epidermidis* for both the noni-supplemented and control calves and the differences of the pairs.

6.2.3.1. Bacterial killing of *E. coli*

Pair	Noni % killing	Control % killing	Difference
1	79	97	-18
2	48.7	70	-21.3
3	45.2	45	0.2
4	90	89	1
5	39	85	-46
6	94	91.7	2.3
7	96.5	95.5	1
8	94.2	95	0.8
9	91.4	40.7	50.7

Table 27. Results percent killing of *E. coli* on day 0

Pair	Noni % killing	Control % killing	Difference
1	82.7	38	44.7
2	45.6	31.75	13.85
3	-10	5.38	-15.39
4	14	-459.81	473.81
5	39	0	39
6	87.4	84.6	2.8
7	90.3	91	-0.7
8	33.8	41.2	-7.4
9	46	44.1	1.9

Table 28. Results percent killing of *E. coli* on day 3

Pair	Noni % killing	Control % killing	Difference
1	35.3	74.1	-39.41
2	20	-20.54	40.54
3	26	7.79	18.21
4	50	43.79	6.21
5	19	-25.53	44.53
6	1	-958.82	959.82
7	5.9	-5	10.9
8	27.3	47.3	-20
9	18.1	-9.1	27.2

Table 29. Results percent killing of *E. coli* on day7

Pair	Noni % killing	Control % killing	Difference
1	25	25	0
2	-10	-98.5	88.5
3	35.9	4.61	31.29
4	80.3	77.3	3
5	54.3	-48	102.3
6	65	44.4	20.6
7	85.6	61.9	23.7
8	42.7	37.3	5.4
9	50	9.1	40.9

Table 30. Results percent killing of *E. coli* on day 14

6.2.3.2. Bacterial killing of *Staph. epidermidis*

Pair	Noni % killing	Control % killing	Difference
1	84.52	76.93	7.60
2	/	/	/
3	73.08	71.70	1.38
4	90.89	90.89	0
5	-418.03	59.02	-477.05
6	/	/	/
7	/	/	/
8	65.96	65.96	0
9	35.11	-134.04	169.15

Table 31. Results percent killing of *Staph. epidermidis* on day 0

Pair	Noni % killing	Control % killing	Difference
1	10.29	-16.18	26.47
2	16.33	36.05	-19.73
3	83.66	74.37	9.30
4	46.02	47.20	-1.18
5	/	/	/
6	12.79	29.07	-16.28
7	4.65	-9.30	13.95
8	42.00	13.33	28.67
9	-80.00	26.67	-106.67

Table 32. Results percent killing of *Staph. epidermidis* on day 3

Pair	Noni % killing	Control % killing	Difference
1	65.43	95.21	-29.79
2	70.60	70.07	0.53
3	64.08	78.50	-14.42
4	51.82	50.36	1.46
5	16.67	8.33	8.34
6	38.64	-15.15	53.79
7	10.61	88.18	-77.58
8	0.00	20.91	-20.91
9	33.64	49.09	-15.45

Table 33. Results percent killing of *Staph. epidermidis* on day 7

Pair	Noni % killing	Control % killing	Difference
1	70.45	78.18	-7.73
2	82.54	78.59	3.94
3	83.73	80.62	3.11
4	35.14	37.84	-2.70
5	-30.77	-103.85	73.08
6	-22.45	23.47	-45.92
7	-2.04	7.14	-9.18
8	79.59	81.63	-2.04
9	78.27	82.65	-4.28

Table 34. Results percent killing of *Staph. epidermidis* on day 14

6.3. Materials

I) Milk replacer

Calf Glo® Insta * Mix Milk Replacer
Milk Replacer to be fed to herd replacement calves

Ingredients	minm/max	
Crude Protein	minm	20.00 %
Crude Fat,	minm	20.00 %
Crude Fiber	max	0.15 %
Ash,	max	9.75 %
Calcium	minm	0.75 %
Calcium	max	1.25 %
Phosphorus	minm	0.70 %
Vitamin A	minm	40000 IU / lb
Vitamin D3	minm	5000 IU / lb
Vitamin E	minm	150 IU / lb

Table 35. Composition of milk replacer (Guaranteed analysis)

II) Calf starter

Middleton Cooperative
18% High Energy Calf Starter – RUM
For Dairy Calves Less Than 3 Months Old

Medicated, active drug ingredient: Monensin 30 g / ton.
For the prevention and control of *Coccidiosis* caused by *E. bovis* and *E. zuernii*

Ingredients	**minm/max**	
Crude Protein	minm	18.0 %
Crude Fat	minm	5.5 %
Crude Fiber	max	6.0 %
Acid Detergent Fiber	max	7.0 %
Calcium	minm	0.6 %
Calcium	max	1.1 %
Phosphorus	minm	0.5 %
Salt	minm	0.4 %
Salt	max	0.6 %
Selenium,	minm	0.4 ppm
Vitamin A	minm	9,000 IU / lb
Vitamin D3	minm	2,000 IU / lb
Vitamin E	minm	50 IU / lb

Table 36. Composition of calf starter (Guaranteed Analysis)

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Selbständigkeitserklärung

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Martin Schäfer