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EFFECT OF *ECHINACEA PURPUREA* & *TRIGONELLA FOENUM-GRAECUM* EXTRACT ON IMMUNITY AND MILK PARAMETERS IN AWASSI EWES

By
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AN ABSTRACT OF THE THESIS OF

Chibli Abou Assi for Master of Science

Major: Animal Science

Title: Effect of *Echinacea purpurea* & *Trigonella foenum-graecum* on immunity and milk parameter in Awassi Ewes.

The aim of this study was to evaluate an experimental *Salmonella Enteritidis* (SE) bacterin and an indirect ELISA system to assess quantitatively the acquired immunity to the vaccine and/or herbal extract of *Echinacea purpurea* (EP) *&* fenugreek; and also to evaluate the above mentioned herbal extract on milk parameters in Awassi Ewes**.** Four treatments of the ewes were included in the experimental design, with six ewes/ treatment. The first treatment (T1) had the controls that were non-vaccinated and non-treated with herbal extract. The T2 ewes were only treated with herbal extract. The T3 and T4 ewes were vaccinated at D1 (initiation of trial) and D10, while the T4 ewes were additionally administered the herbal extract. Blood was collected from the jugular vein of all ewes at D1, D10, D21 and D45 and milk was collected at D1, D10, D21, and D45. The construction of the vaccine and the ELISA are detailed within the manuscript. The ELISA was able to detect quantitatively the significant acquired primary and secondary immunity to the vaccine in T3 and T4 ewes, compared to their low level of background immunities at initiation the experiment ($p<0.05$). In addition, the ELISA detected the absence of seroconversion at all blood sampling times ($p>0.05$) in T1 control ewes, and in the T2 ewes that were given only the herbal extract ($p>0.05$). Moreover, the ELISA was able to uncover the significant seroconversion of secondary immune response in T4 ewes at D21 compared to that at D10 ($p<0.05$), and the absence of significant seroconversion of secondary response in T3 ewes. With respect to milk parameters which are Milk decrease%, SNF, Protein, Density, Lactose, and Fat, there was no significant differences among the experimental groups ($p>0.05$); although vaccinated and herb administered ewes showed higher values for the mentioned milk parameters. This is the first work in literature that reports the need to supplement the vaccination by the experimental SE bacterin with daily oral intake of 250 mg and 610 mg of EP and Fenugreek respectively,effective the first vaccination day and up to 45 days, for obtaining a statistically significant seroconversion.

Keywords: Awassi ewes, *Echinacea purpurea* (EP), ELISA, Experimental SE vaccine, *Salmonella Enteritidis* (SE).

CONTENTS

TABLES

ABBREVIATIONS

ELISA : Enzyme-Linked Immunosorbent Assay

EP : *Echinacea Purpurea*

SE: *Salmonella Enteritidis*

T : Treatment

D : Day

Ig:Immuno globulins

PBS :Phosphate Buffered Saline

BSA : Bovine Serum Albumin

OD : Optical Density

SEM :Standard Error of Means

ABTS: 2,2-azino-di 3 ethyl- benzthiazoline-6-sulfonate

PFGE :Pulsed-Field Gel Electrophoresis

TESSy: The European Surveillance System

RASFF: Rapid Alert System for Food and Feed

LTCF: Long-Term Care Facility

PFGE: Pulsed-Field Gel Electrophoresis

DPH's: Department of Public Health

LPS: Lipopolysaccharides

HRP: Horse Radish Peroxidase

O: somatic

H: flagellar

Vi: capsular antigens

E. coli: Escherichia coli

MHC: Major Histocompatibility Complex

CB: Cannabinoid

mRNA: Messenger RNA

ACTH: Adrenocorticotropic Hormone

TNF: Tumor Necrosis Factor

NF-Kb: Nuclear Factor Kappa-light-chain-enhancer of activated B cells

ºC: degrees Celsius

MDa: mega daltons

EU: European Union

µg: Microgram

µl: Microliter

µM: micrometer

SUS: Saybolt Universal Seconds

SDS: sodium dodecyl sulfate

W/V: weight / volume

EC_{50}: Half maximal effective concentration

JNK: c-Jun N-terminal kinases (JNKs)

ATF-2: Activating Transcription Factor 2

CREB-1CAMP: responsive element binding protein 1

cAMP: Cyclic adenosine monophosphate

TLR4: Toll-like receptor 4

LPS: Lipopolysaccharides

PBMCs: peripheral blood mononuclear cell

IL: Interleukin

IFN-γ: Interferon gamma

CD: cluster of differentiation

EchNWA: *Echinacea Purpurea* L. Moench

CHAPTER I

INTRODUCTION

Salmonella Enteritidis (SE) is the most common serovar in poultry and other wide spectrum of hosts (Santos et al., 2011). The use of Salmonella-contaminated chicken litter, as a soil conditioner, is a common practice in most developed and developing countries, including Lebanon (Barbour et al., 1999; Chen et al., 2014). It is documented that SE organisms persisted in the litter for the whole period of 26 months sampling, and in soil for 8 months, and caused the infection of different mammalian species that were in direct contact with the contaminated soil (Davier and Breslin, 2003; Nicholson, 2005).

The SE is the causative agent of the most common human salmonellosis in Europe, originating from the consumption of contaminated animal products (Peters et al., 2007). Vaccine development against economic diseases in domestic ruminants is in progress, due to emergence of new escape mutants, and pathogens adapting to wide spectrum of hosts (Lewis et al., 1989; Barbour et al., 1996). More specifically, Salmonella bacterins, protecting against different challenging serovars to animals, are in continuous development around the world (Barbour et al., 1993; Kassaify et al., 2011). The immunopotentiation of vaccines by natural products is another new trend in research, targeting an improvement in seroconversion that enhances protection against specific diseases (Qiu et al., 2007; Bagherwal and Machawal, 2013). The *Echinacea purpurea* (EP) root powder is rich in caffeic acid derivatives (phenolic compounds), alkamides, and polysaccharides. This herb was able to stimulate the lymphocyte proliferation in response to sheep red blood cells in a mice model (Skopinska-Rozewske et al., 2011); in addition, the ingestion of Echinacea in rats led to a significant increase in the level of antigen specific-IgG,

with an increase in this isotype equivalent to 34.6 % compared to the control rats that were deprived of this herb (Rehman et al., 1999).

The assessment of the acquired humoral immunity to vaccination and/or immunopotentiators required a development of quantitative system that can determine the primary and secondary immune response specific to the antigens used in developing the vaccines. The main disadvantage of using commercial kits is the lack of specificity, since the coating antigens might differ from those of the newly emerging field strains included in the bacterin (Meng et al., 2012). The development of an ELISA system for the assessment of vaccine efficiency and/or natural and synthetic immunopotentiators, is a golden standard tool that has been used frequently in the last decades (Barbour et al., 1996; Balamurugon et al., 2017; Fan et al., 2012). In developing ELISA protocols, a checkerboard titration is performed for optimizing the quantities of various reagents included in the system, such as the dilution of the serum sample, the conjugate, the blocking substance included in the serum diluent and coating buffer, and the wavelength at which the maximum absorbance of the developed color is read (Shang et al., 2010; Mahgoub, 2014).

In addition to the role of herbal extracts as immunopotentiators, herbal inclusion in animal diets has been extensively used to enhance the performance parameters. Fenugreek seeds were intensively used worldwide in ruminant diets for their galactogenic properties. Fenugreek belongs to the family Leguminosae, its seeds contain; alkaloids, flavonoids, saponins, amino acids, tannins and certain steroidal glycosides as well as proteins (Yadav et al., 2011). Saponins are chemical compounds found in some plants; they were used as natural detergents. In the past Arab women's used to depend on fenugreek herb in order to increase their breast milk. Fenugreek is mainly used for its seeds that are valuable nutritional components. Diocin is a

natural saponin found in Fenugreek and has a structural similarity to estrogen, which leads to an increased release of growth hormone (GH) by binding to the receptors on pituitary cells that recognize the GH releasing hormone. This, in turn, results in an increase in milk secretion (Graham et al., 2008). In addition, Fenugreek stimulates the galactogenic hormone, prolactine, which further increase the milk production.

To our knowledge, this is the first research aiming at evaluating an experimental *Salmonella Enteritidis* bacterin and a related indirect ELISA system to assess quantitatively the acquired immunity in Awassi ewes specific to the vaccine homologous antigens, in absence or presence of *Echinacea purpurea* dried roots supplementation.

CHAPTER II

LITERATURE REVIEW

A. Immunopotentiators

Immunopotentiators, also known as immunostimulators are substances or molecules that stimulate or enhance the activity of the immune system and its components (Kumar et al., 2011). There are two main categories of immunopotentiators:

1. Specific immunopotentiators provide antigenic specificity in immune response, such as vaccines or any antigen.
2. Non-specific immunopotentiators act irrespective of antigenic specificity to augment immune response of other antigens or stimulate components of the immune system without antigenic specificity, such as adjuvants and non-specific immunostimulators.

With the development of modern intensive farming, there is an increased risk of animals being infected with various diseases. Immune function directly affects the animal health and production capacity, thus affecting the quality of human life. In order to prevent animal diseases, to promote animal growth and improve feed efficiency; various methods are being employed such as use of large number of antibiotics and chemical synthetic drugs or steroids being applied as a feed additive. However, the improper use of antibiotics and drugs leads to decreased immunity in the animal, increasing the chances of infection and poor survival. One of the best alternatives for these synthetic drugs is the use of phyto-nutrients that are plant based nutrient

supplements that stimulate the natural immunity of the animals and empower them with better survival and fitness. Immunostimulatory effects of various phytogenic compounds have been extensively investigated in human and laboratory trials, which indicate a potential of their beneficial effects in poultry and other livestock species. A study by Masamha et al. (2010) was conducted to study the effect of garlic (*Allium sativum*) extracts on gastrointestinal nematodes in sheep in Zimbabwe. The effect of garlic extract was confirmed to a synthetic dewormer named Valbazen and it was found that the garlic extract was as effective as the commercial dewormer in controlling the nematodes and also proved to act as an immunopotentiator by controlling the nematodes for a long period without any side effects. Moreover the garlic extract was found to activate the innate immune system of the sheep against the parasitic nematodes. These results were similar to the effects of garlic extract on nematode control in sheep observed by Allen et al., in 1998.

Echinacea purpurea (EP) (family *Asteraceae*) belongs to the group of phytogenic immunostimulants that help in establishing and strengthening of para-immunity and is reported to possess a number of pharmacologically active substances (Akhtar et al., 2003). Echinacea is popular herbal immune-stimulator in North America and Europe, well known for its beneficial effects on immune system and as natural remedy in many diseases (Nasir and Grashorn, 2006; 2009a).

Echinacea and its different preparations contain a variety of active substances like alkamides, glycoproteins, polysaccharides, phenolic compounds, cinnamic acids, essential oils and flavonoids (Barrett, 2003; Zhai et al., 2007; Nasir and Grashorn, 2009a). These substances are proven to have great potential in the treatment of various diseases and are beneficial in

improving immunity (Woelkart and Bauer, 2007). While trying to elucidate the mechanism of action, it was found that solutions made from herb and root EP powders produced defined reproducible macrophage activity and proved anti-inflammatory and antioxidant properties (Rininger et al., 2000). Nasir (2009) studied the effects of solution of EP as oral feed on broilers; he found significant weight gain and overall improvement in the health status of broilers fed with the EP juice. He concluded that beneficial effects of Echinacea supplementation on broiler performance, health and immunity can be obtained by intermittent application of EP through drinking water.

A study by Doaa et al., (2009) was performed to investigate the effect of using Echinacea extract, as immuno-stimulating feed additive, on milk production, milk composition, udder health and immune response of Zaraibi goats. Immulant pills containing *Echinacea purpurea* extract were given orally once daily over two weeks to the goats. They found that the Echinacea pills had beneficial effects on the mammary glands, increased the quality and hygiene of the milk and also improved the goat immune response, leading them to suggest that Echinacea extract (525 mg/day/head) when supplied to goats would yield better economic return.

A Chinese patent (ID -CN102429122 B) consisting of a mixture of Astragalus, Rhizoma, malt and Echinacea has been proven to have immunopotentiating effects in sheep. They found that this combination of plant extracts increased immune responses in sheep, better weight gain, and better immunity to diseases. Another patented invention of ID CN102100784 A has shown EP was the main ingredient given to chicken. The results indicated that when Echinacea was added to drinking water in a concentration of 0.15% and 0.3%, chicken peripheral lymphocyte proliferation improved and moreover, throughout the experimental period of three weeks, there

was no chicken death in each group. These findings clearly established the role of EP as a highly potent immunostimulator.

Another study by Sgorlon et al. (2012) in Sarda sheep was to investigate the regulation of gene expression in blood leukocytes during ACTH-induced cortisol release and the effect of dietary administration of botanicals to counteract the evoked response in polymorphonucleate cells. They found that oral administration of Echinacea extracts to the sheep induced beneficial transcriptosome changes and hence concluded that Echinacea could serve as a promising feed supplement in ruminants to cope with conditions associated with increased concentrations of plasma cortisol.

A study by Reklewska et al., 2004, involved the use of Echinapur preparation, containing *Echinacea purpurea* extract, to promote milk lactoferrin secretion, and as a result of its antibacterial properties to reduce inflammatory changes in the goat's mammary gland. They found out that the administration of the Echinacea extract for two weeks to the goats resulted in a marked increase in milk yield as well as lactoferrin content. This pioneering study proved the effectiveness of Echinacea as an immunopotentiator that reduced the inflammatory changes in goats mammary glands significantly. Their study proved that the Echinacea preparation possessed anti-bacterial, anti-viral and immuno-stimulating properties.

B. Components of *Echinacea purpurea* responsible for immunopotentiation in different animal models

The use of *Echinacea purpurea* (EP) preparations for the treatment of various infections especially wound infections and healing, has been well documented. Published studies indicated that it has immunostimulatory properties. As such, it has been used in prophylaxis and therapy of various infections mainly the respiratory tract, in animals and humans. The Echinacea products have been known for a long time as scavengers for free radicals. For this reason, they enhance the efficacy of the immune system and are, therefore, incorporated in many dietary supplements worldwide. Roots and aerial parts of EA are usually used and they are derived from three species: *Echinacea purpurea*, *Echinacea angustifolia* (EA) and *Echinacea pallida*. The following are the active compounds that were isolated from Echinacea: caffeic acid derivatives (phenolic compounds), alkamides and polysaccharides. The caffeic acid derivatives include several components, such as: caftaric acid, chlorogenic acid, caffeic acid, echinacoside and chichoric acid. On the other hand, a major phenolic compound in *E. purpurea* is Chicoric acid. This compound is present at low levels in *E. angustifolia* and *E. pallida*. Echinacoside is the main phenolic compound in *E. angustifolia* and *E. pallida*, but not in *E. purpurea*.

In Macrophages, the presence of alkylamides in EP resulted in the activation of cannabinoid receptors thus making them possess more affinity for CB2 (cannabinoid) which is dominantly expressed on the immune cells as compared to CB1 (Pertwee et al., 2006). It was reported that a solution of alkylamides resulted in the activation of CB2 on both the monocytes and macrophages with an EC_{50} of less than 1μM (Gertsh et al., 2004). It has also been demonstrated that certain alkylamides induced TNF-α release in macrophages and monocytes (Gertsch et al.,

2004). This was mediated by NF-κB activation, JNK/ATF-2 and CREB-1 as intermediates, and is additionally cAMP dependent (Pertwee et al., 2006). Feeding rats with alkylamides at 12 mcg/kg resulted in TNF-α activation and is produced in isolated macrophages through TLR4 mechanisms (Goel et al., 2002; Sullivan et al., 2008). For this reason, EA alkylamides (has a modulatory potential on macrophages because the overall NF-kB activation in macrophages treated with both LPS and Echinacea is less than that seen with LPS alone (Sullivan et al., 2008; Stevenson, 2005). A study, in which endotoxin-free EP was employed, showed a decrease in TNF-α release by 24% from PBMCs from subjects fed with 4mL of Echinaforce for 3 days, followed by 10mL for 3 days (Benson et al., 2010). In an in vitro comparative study, it was demonstrated that TNF-α activation was more pronounced in the case of EA as compared to pallid (Senchina et al., 2011).

The effect of EA on interleukins showed that they consistently resulted in the induction of both IL-8 and IL-6 when the dose is changed *in vitro* in leukocytes (Kapai et al., 2011). The reduction of IL-1β secretion from PMBCs was noted using Endotoxin-free EA while potentiating IL-10 by approximately 13%. This was associated by a weak induction of IFN-γ and IL-8 (as measured in cells obtained from persons who administered 4mL Echinaforce for 3 days and 10mL for another 3 days) (Ritchie et al., 2011). In a recent clinical study, using 10 healthy individuals who administered herbal syrup once daily for a period of one month, on the immunomodulatory effects of a triply standardized *Echinacea angustifolia* root extract (Polinacea®).The results indicated an important role for the standardized *Echinacea angustifolia* root extract in cytokine expression. In lympho-monocytes an up-regulation of the mRNA levels of IL-2 and IL-8 and the down regulation of the mRNA levels of the pro-inflammatory cytokines TNF-α and IL6 were noted (Dapas et al., 2014). In addition, a neutral and weakly acidic water-

soluble extract from EP (L.) Moench polysaccharides, but not phenolic compounds have dose-related adjuvant effects on human T-cell cytokine responses characterized by enhancing and suppressive effects that are regulated by T-cell density (Fonseca et al., 2014).

In T-cells, both stimulatory and inhibitory effects were noted in the case of EA on T-lymphocytes. For instance, in response to the presence of a mitogen (*Phaseolus vulgaris* haemagglutinin) in mice, the different Echinacea species generally stimulated lymphocyte proliferation (Skopińska-Rózewska, 2011) in response to sheep red blood cells (mice); such a lymphocyte proliferation was noted in vitro with alkylamides at 50mcg/ml due to the stimulation of interferon IFNγ production in anti-CD3-treated murine T-cell cultures, and in vivo with a notable increase in CD4+ lymphocytes (Zhaietal, 2007; Sasagawa et al., 2006; Mishima et al., 2004; Morazzoni et al., 2005). In another study, Echinacea juice (leaf) supplementation caused a slight suppression of 6% in T-cell levels with a decrease in T-cell release of IL-2, TNF-α and IL-1β (Zhaietal, 2007; Sasagawa et al., 2006; Schwarz et al., 2005) with the possibility of reduced antigen uptake from dendritic cells by T-cells (Benson et al., 2010). In a recent study, Echinacea alkylamides suppressed IL-2 secretion by stimulated T cells, and this effect was significantly reduced upon the oxidation of the alkylamides to carboxylic acids and hydroxylated metabolites indicating the emphasis on the importance of considering the influence of liver enzyme metabolism when evaluating the immunomodulatory effects of alkylamides (Cech et al., 2014).

The effects of EA on Dendritic cells are unclear. Those are antigen presenting cells that mediate innate and adaptive immunity and act as a presenter of antigens to T-cells for recognition

(Banchereau et al., 2000). As a result, their activation and proliferation result in a greater antigen recognition and adaptive immunity (in response to sickness) (Banchereau et al., 2000).

The basic root extract (containing polysaccharides, mostly glucitol acetate and mannitol acetate) can raise the levels of CD86 and CD54 positive cells in a dose-dependent manner, increasing from 10% to 25% and 27% (CD86) and from 12% to 30% and 32% (CD54) (Benson et al., 2010). Such an extract reduced the content of CD86, CD54, and MHC II relatively, as a result of a high induction of CD11c+ BMDCs. In a different study, an induction of CD54 was seen when using an ethanolic root extract alongside a general stimulatory effect (Wang et al., 2006).

The leaf extract at a concentration of 50mcg/ml increased the levels of CD11c+ BMDCs from 75% in the control to 94% and to 100% when applied at a concentration of 150mcg/ mL. However, the root extract was less effective in the case of other positive cells (CD86, CD54, MHC II) (Benson et al., 2010).

Differential effects were shown in CD83+ cells which were stimulated with a butanolic extract (both stems and roots) and suppressed when using an ethyl acetate fraction (Wang et al. 2008).

Both the root and leaf extract appeared to significantly reduce antigen uptake of dendritic cells and inhibited the interactions between dendritic cells and CD4+ T cells (Benson et al., 2010).

In vivo studies indicated that oral ingestion of extracts of EA has anti-inflammatory effects as determined by the granulation formation test (28.52%), paw edema (48.51%), and ear edema (44.79% inhibition) (Yu et al., 2013). The presence of low concentrations of Cynarin in Echinacea which is known to be immunosuppressive precludes its effects (Dong et al., 2009). The results of a recent study where they tested the hypothesis that in vitro pro-inflammatory effects of *E. purpurea* crude extracts could be caused by bacterial endophytes (bacteria living

asymptomatically within the plant tissues) indicated clearly that bacterial endophytes play a role in the in vitro pro-inflammatory activity of ethanolic *E. purpurea* extracts. Also, they identified a previously unreported anti-inflammatory constituent of *E. purpurea*, 4-[(2-methylbutyl) amino-4-oxo-2-butenoic acid (Britton et al., 2014).

Considering the effects of EP on adaptive immunity, it was reported that the ingestion of EP by rats led to a significant Increase in the levels of antigen specific immunoglobulins M and G leading to increase of IgG to 34.6% as compared to control on day 20 (Rehman et al. 1999).

C. Fenugreek

Fenugreek plays a role in activating prolactin secretion. The lactogenic hormone, prolactin, secreted by the anterior pituitary is critical to the establishment of lactation, milk macronutrient content and milk production. The concentration of circulating prolactin increases during pregnancy so that by the end of gestation, levels are 10 to 20 times over normal amounts. However, prolactin is prevented from exerting its effect on milk secretion by elevated levels of progesterone. Following clearance of progesterone and estrogen at parturition, milk secretion begins. The minimal hormonal requirements for normal lactation to occur are prolactin, insulin and hydrocortisone. Prolactin stabilizes and promotes transcription of casein mRNA; may stimulate synthesis of alpha-lactalbumin, the regulatory protein of the lactose synthetase enzyme system; and increases lipoprotein lipase activity in the mammary gland. Prolactin levels decrease as lactation is established but nursing stimulates prolactin release from the pituitary which promotes continued milk production. Prolactin is secreted into milk at levels representative of the average circulating concentration.

1. Effect on milk production

Tomar et al. (1996) studied the influence of supplementation of fenugreek at the level of 200 g/day with Murrah buffaloes. Milk yield was significantly higher in treated group than control. However no effect on milk components was detected.

Allam et al. (1999) studied the effect of fenugreek seed at the level of 500 mg/kg LBW in Zaraibi goat and found an increase in average daily milk yield and fat corrected milk. However, total solids, crude protein and lactose percentages were alike.

Al-Shaikh et al. (1999) supplemented 0, 25 and 50 per cent fenugreek seeds in the concentrate mixtures in the diet of goats and detected higher milk yield and fat per cent at 25% levels.

Kholif et al. (2001) studied the effect on lactating goat by supplementing 10 g/ day of fenugreek seeds. It increased milk yield, total nitrogen ($P < 0.05$), soluble nitrogen and salt contents ($P < 0.01$). In another study by Kholif and Shewy (2004), it was found that fenugreek seed supplementation in Baladi 27 goats increased milk fat and protein yields significantly but total solids, milk proteins, fat, ash, lactose and SNF were not affected.

2. Effect on Blood Parameters

Tomar et al. (1996) reported that blood composition was not affected by fenugreek seed supplementation. Similarly, no significant differences were found in plasma total protein, albumin, globulin, cholesterol, glucose and total lipids by supplementation of fenugreek seeds

(Al-Shaikh et al., 1999). Higher blood glucose ($p<0.05$) and lower cholesterol and total lipids were observed in lactating buffaloes as compared to control(El-Alamy et al., 2001).

3. Effect on feed intake and digestibility

El-Nor (1999) did not observe a significant effect on dry matter intake (DMI) on fenugreek seed supplementation. Digestibility coefficients of DM, organic matter, crude protein and nutritive value as total digestible nutrients (TDN) and starch equivalent were improved by fenugreek seed supplementation at the levels of 500 mg/kg in Zaraibi goats (Allam et al., 1999).

Sahin et al. (2003) reported no effect in daily live weight gain and feed intake ($P < 0.05$) in Awassi lambs fed fenugreek seeds at the level of 0, 2, 4 and 8 per cent in diet.

4. Effect on immunity

Aqueous extract (50, 100 and 250 mg/kg b.w.) of fenugreek seeds were evaluated in male Swiss albino mice for 10 days. Fenugreek showed stimulatory effect on immune functions as indicated by cellularity of lymphoid organs, delayed type of hypersensitivity response, phagocytosis and lymph proliferation (Bin-Hafeez et al., 2003).

5. Antioxidant activity

Enhanced lipid peroxidation associated with the depletion of antioxidants in liver, kidney and pancreas in diabetic rats were normalized on supplementation of fenugreek seed powder in the diet for 30 days at a dosage of 2 g/kg b.w. (Anuradha and Ravikumar, 2001).

6. Gastro protective activity

An aqueous extract and a gel fraction of fenugreek seeds were shown to protect the gastric mucosa more efficiently than omeprazole in rats from HCl-ethanol induced gastric ulcers (Pandian et al., 2002)

D. Salmonellosis

The Gram-negative rod-shaped strains of the genus Salmonella display phenotypic characteristics of the family Enterobacteriaceae. Salmonella serovars are generally motile, straight rods with flagella that grow on nutrient agar in aerobic or anaerobic conditions. Salmonella are generally non-lactose fermenters or slow lactose fermenters and most systems for the detection of the organism are based on this property. However, some peculiar serovars rapidly ferment lactose. Identification of Salmonella isolates is based on biochemical tests. The chemical structure of Salmonella antigens has been determined. Serovars are characterized on the basis of somatic (O), flagellar (H) and capsular (Vi) antigens (LeMinor and Popoff, 1987). Serogroups have been identified on the basis of the O antigens with each serogroup having a group-specific O antigenic factor. Serovars are determined on the basis of the combination of O and H antigens. Vi antigen is a capsular polysaccharide found in S. typhi, S. paratyphi C and S. dublin. In addition, several genotypic typing methods such as ribotyping, pulsed-field gel electrophoresis (PFGE) and amplified fragment length polymorphism (AFLP), and PCR are used to differentiate isolates in epidemiological studies. Phage typing, biotyping, drug resistance and plasmid profile analysis may be used to identify isolates beyond the level of

serovar. Phage typing is limited to a few serovars, such as S. typhi, S. typhimurium, S. dublin, S. enteritidis, S. heidelberg and S. schottmuelleri (Clarke and Gyles, 1993).

The genus Salmonella contains a large group of bacteria that cause many infectious diseases in humans and animals such as mammals, birds, fish and reptiles. Domestic animals suffer a lot from these organisms that causes significant morbidity and mortality especially for the new born and pregnant animals. It also affects sheep subjected to stressful conditions such as those encountered during animals shipping (Mukkur and Walker, 1992, plagnemann, 1989; Wray et al., 1991). In addition salmonella may infect humans through the food chain (Nabbut et al., 1982).

The disease is called salmonellosis, although the term paratyphoid may be used (for example, swine paratyphoid) or pullorum disease (S. pullorum) and fowl typhoid (S. gallinarum) in poultry. Salmonellosis is spread all over the world, but the most infected areas are the ones that follow the intensive systems of animal production. These systems depend on the import of animal feed to the countries with intensive animal production systems and, this results in an international widespread outbreak of salmonellosis in animals and human as such.

Recent gene-sequencing analyses have indicated that *Salmonella* and *Escherichia coli* might have diverged from a common ancestor 120-160 million years ago, coincident with the origin of mammals (Selander et al., 1996). In 1880, Eberth observed the typhoid bacillus in spleen sections and mesenteric lymph nodes of a patient that died from typhoid. In 1886, Salmon and

Smith isolated *S. cholerasuis* from pigs. Since then, more than 2400 different Salmonella serovars have been identified, and new serovars are being described each year.

Salmonella is a member of the family Enterobacteriaceae. Classification is still complicated and it has been modified several times in the recent years. The binominal nomenclature has been widely used, based on recommendations of the International Code of Nomenclature of Bacteria for recognition of a genomic species. According to these recommendations, the genus Salmonella is divided into two species, S. enterica and S. bongory. S. enterica is further divided into six subspecies (S. enterica subsp. enterica, S. enterica subsp. salamae, S. enterica subsp. arizonae, S. enterica subsp. diarizonae, S. enterica subsp. houtenae and S. enterica subsp. indica). Although this nomenclature is not yet validated, it is scientifically based and less confusing than other proposals. Serovar names are no longer considered as species names. For example, S. typhimurium is now called S. enterica subsp. enterica serovar Typhimurium, although some researchers still prefer to use the simpler form Salmonella serovar Typhimurium (or Salmonella Typhimurium). Only serovars of S. enterica subsp. enterica are given names (usually based on the name of geographic location of their first isolation). Other serovars are identified only by the subspecies, followed by their O: H antigenic formula.

S. enteritidis affects chickens and pheasants, but does not seem to affect fertility, hatchability or egg production. It can cause clinical disease in chicks less than 1 week of age. S. enteritidis infection in older broilers causes unevenness and stunting.

Salmonella abortisovis affects mainly sheep; it causes ewe abortion especially for the ones newly introduced to the flock. It leads to economic losses characterized by abortions, stillbirths, and illness in lambs infected at birth. Infection from *Salmonella Abortusovis* can be found worldwide, but is mostly common in Europe and Western Asia. Infections have been reported in different countries such as France, Spain, Germany, Cyprus, Italy, Switzerland, Russia, and Bulgaria. *S.abortusovis* it has been occasionally isolated from goats and rabbits; while mice and rabbits can be experimentally affected. Antibody has been found in red deer. This disease is caused by Salmonella enterica subspecies enterica serovar (serotype) Abortusovis. Salmonella Abortusovis, a member of the Enterobacteriaceae, is a short, aerobic, Gram–negative rod.

E. Salmonella Enteritidis infections in humans

Salmonella serotype Enteritidis (SE) is one of the most common serotypes of Salmonella bacteria reported worldwide. During the 1980s, SE emerged as an important cause of human illness in the United States. Eggs have been the most common food source linked to SE infections. SE can be inside perfectly normal-appearing eggs. If eggs contaminated with SE are eaten raw or lightly cooked (runny egg whites or yolks), the bacterium can cause illness. Since the early 2000s, poultry has also been found to be a common food source for SE infections (Hrivniakova, 2011). Multiple other, less frequently identified sources include raw milk, pork, beef, sprouts, and raw almonds. International travel and contact with reptiles have also been associated with SE infection. A person infected with the Salmonella bacterium usually has fever, abdominal cramps, and diarrhea beginning 12 to 72 hours after consuming a contaminated food or beverage. The illness usually lasts 4 to 7 days, and most persons recover without antibiotic

treatment. However, the diarrhea can be severe, and the person may be ill enough to require hospitalization. The elderly, infants, and those with impaired immune systems may have a more severe illness. In these patients, the infection may spread from the intestines to the blood stream, and then to other body sites and can cause death unless the person is treated promptly with antibiotics (CDC, 2010).

1. Salmonella outbreaks

S. Enteritidis is the most commonly detected serovar in human salmonellosis in Europe (Peters et al., 2007). From 2007 to 2013, 328 537 S. Enteritidis cases were reported to the European Surveillance System (TESSy) (mean number per year 46 934, range 29 350 to 83 376) by 27 countries, with Germany and the Czech Republic together reporting 52% of all cases. Of all cases with available information, 43% (n=139 090) were under 15 years, 51% (n=168 725) were female and 90% (n=245 479) were acquired within the reporting country. Symptom onset of cases was distributed across the year, with a peak from July to September. In the first quarter of 2014, 2 076 cases were reported to TESSy. Enteritidis is the predominant serovar associated with the Salmonella outbreaks (EFSA, 2014). In 2012, S. Enteritidis accounted for 179 outbreaks and 2 177 human cases (37.6% of all cases in Salmonella outbreaks). Most of these S. Enteritidis outbreaks were attributed to eggs and egg products. In the same year, egg and egg products were implicated in 168 outbreaks (22%) out of 763 outbreaks reported at EU level, of which 93.5 % were caused by Salmonella spp. The majority of these outbreaks were associated with S. Enteritidis (66.7%), as in previous years (Jan Mohammed et al., 2011).

In 2014 so far, twenty-six notifications of S. Enteritidis have been reported to RASFF (Rapid Alert System for Food and Feed), mostly associated with poultry meat (n=19), but also with eggs (EFSA, 2014). Styles et al., 2013 reported that a multiagency outbreak investigation was initiated in a long-term care facility (LTCF) in Connecticut and identified a total of 21 possible salmonellosis cases; nine were culture-confirmed Salmonella serotype Enteritidis with an indistinguishable pulsed-field gel electrophoresis pattern (PFGE). This report describes the epidemiologic, environmental, and laboratory investigation conducted as part of DPH's (Department of Public Health) response. Undercooked raw shell eggs were the likely source of infection. This investigation reemphasizes the vulnerability of certain populations to severe illness from Salmonella and further stresses previous recommendations in the literature to use only pasteurized egg products in long-term care and other healthcare facilities.

F. Development of ELISA protocol for evaluation of serum titers of immunized/immunopotentiated animals

The enzyme-linked immunosorbent assay (ELISA) is a powerful immunological method for detecting specific antibodies or specific antigens following infection, immunization and or immunopotentiation. Commercial kits are available, providing high sensitivity and repeatability in assessing animals' antibody titers against various bacterial and viral pathogens (Kim, 2010; Bang et al., 2012). The main disadvantage of using commercial kits is the lack of specificity, since the coating antigens might differ from those of the newly emerging local field strains of various pathogens. Escape mutants of animal pathogens are in continuous emergence under the vaccination pressure or with the uncontrolled continuous use of drugs in livestock and poultry farms, especially in the developing countries (Saif et al., 2008).

The development of ELISA plates for the assessment of vaccine efficiency and/or immunopotentiating effect of natural and synthetic products against specific antigens of local pathogens is a golden standard tool that has been used frequently in the last decades (Zoubiane 1993, Balamurugan et al., 2007, Fan et al., 2012). In developing ELISA protocols, a checkerboard titration is performed for optimization of various reagents used such as: the type and amount of the coating antigen, the working dilution of the serum samples and the conjugate, the type of blocking substance in the serum diluent or the coating buffer, and the wavelength at which the absorbance is read (Shang et al., 2010; Liu et al., 2011, Mahgoub, 2014).
The type of coating antigens can vary from whole organisms to specific antigens that could be either cloned in specific plasmidic vectors, or separated by electrophoresis and or dialysis. Various works on animal viral diseases such as parvoviruses, avian influenza, avian hepatitis,

avian syncytial virus, cloned a specific gene segment in various E. coli or other bacterial plasmid, Rinderpest and Pest des Petits Ruminants viral isolates were propagated in Vero cell culture, purified and used as whole organisms to coat the ELISA plates (Zoubiane, 1995; Balamurugan et al., 2007; Pererat and Murray, 2009; Fan et al, 2012; Zhao et al., 2013; Wang et al., 2013). A number of ELISAs for detecting anti-Salmonella antibodies have been described based on lipopolysaccharides (LPS) (Veling et al., 2000; House et al., 2005) and flagellar antigens (Veling et al., 2000; Gast et al., 2002; Dalby et al., 2005). On the other hand, using whole Salmonella organisms is very common in coating ELISA plates such as in the works of Bang et al (2012) on *Salmonella Typhimurium* in rabbits, and Zoubiane (1995) on *Salmonella Enteritidis* in sheep. Bang et al. (2012) successfully coated ELISA plates with whole *Salmonella Typhimurium* organisms following their inactivation with formalin, while Zoubiane (1995) separated and charged the *Salmonella Enteritidis* antigens with 1/29.1 SDS/antigenic protein amount ratio before plate coating.

In standardizing the amount of coating antigens, the amount of antigenic protein for plate coating, in developing ELISA plates, can vary from 24µg to 700 µg/ well. Literature reported that lower rates of antigens (24 to 400 µg/ well) usually results in optimal reading of absorbance. Plates could be incubated, after the addition of the coating antigens, at either 4˚C or 37˚C overnight (Zoubiane, 1995, Balamurugan et al., 2007; Pererat and Murray, 2009; Fan et al, 2012; Zhao et al., 2013). The optimal *Salmonella Enteritidis* antigenic protein level for plate coating, as concluded from the work of Zoubiane (1993), was 48µg/100µL/well.

Standardization of the sera and conjugate dilution rates, for the assessment of serum antibodies against animal diseases, resulted in adequate dilution rates ranging between 1:50 to 1:200 for

sera and between 1:1000-1:10000 for Horseradish Peroxidase (HRP) labeled conjugates (Zoubiane, 1995, Balamurugan et al., 2007; Pererat and Murray, 2009; Fan et al, 2012; Zhao et al., 2013). In standardizing ELISA protocols for Anti-Salmonella sera titer assessment, a 1:100 dilution of the serum and a 1:2000 dilution of the conjugate are found to be the most adequate for absorbance reading at 450 nm wavelength (Zoubiane 1995, Pererat and Murray, 2009; Bang et al., 2012).

There is a wealth in literature emphasizing the use of blocking agents in the primary and secondary antibodies. The presence of these blocking agents is primordial in developing ELISA protocols, since they will coat the active sites of the plates, avoiding false positive readings resulting from the attachment of antibodies to the side walls of the wells (Dufour-Zavala et al., 2008). Most of the blocking agents used in ELISA protocol development include skim milk in various concentrations such as 2.5 and 5% in Phosphate Buffered Saline (PBS) (Pererat and Murray, 2009; Bang et al., 2012), 5% Fetal Bovine Serum in PBS (Fan et al., 2012) and 1% Bovine Serum Albumin in PBS (Mahgoub, 2014).

1. Preparation of Positive and Negative sera controls

Positive and negative control sera are primordial for the development of ELISA protocols to assess animal antibody levels against specific pathogens. Positive control sera are usually prepared by pooling the sera of immunized/immunopotentiated animals; two to three weeks post priming, or boosting of the experimental animals. The negative control serum is usually prepared by pooling sera of control animals that are deprived of any immunization/immunopotentiation at

the same positive sera collection date (Gast et al., 2002; Dalby et al., 2005). Three to six replicates of the pooled positive and negative controls are run to ensure the efficiency and repeatability of the developed ELISA protocol (Kim, 2010; Bang et al., 2012, Fan et al., 2012). ELISA mean OD values of the negative controls range between 0.1 and 0.25 while that of the positive control serum ranges between 0.7 and 1.13, as reported in literature (Zoubiane, 1995, Balamurugan et al., 2007; Pererat and Murray, 2009).

CHAPTER III

MATERIALS AND METHODS

A. Experimental Salmonella Enteritidis (SE) vaccine

The experimental SE vaccine is modified from our previous documentation (Barbour et al., 1993) that attempted to protect against predominant phage types of SE in the USA including phage type 8,13a, and 23. The SE strain used in this new bacterin is of higher virulence due to its invasive nature, caused by the presence of a high size plasmid of 38 MDa, known to exist in highly virulent SE strains of phage type 4 (Powell, 1995). Briefly, the SE bacterin contained in its aqueous phase an amount of $4.8x10^8$ colony forming units per ml of SE cells, inactivated by 0.3 % formalin, and emulsified into equal volume of sterile 40 SUS mineral oil, supplemented with 4.3%and 0.1 % (W/V) of Arlacel C and Tween 80, respectively.

B. Construction of an optimized ELISA

The construction of an optimized ELISA for quantification of the ewes-serum antibodies, specific to antigens of the SE that acquired a plasmid of 38 MDa size, was based on a checkerboard, targeting to result in significant differences in absorbance of the developed color between replicates of SE-negative and SE-positive control sera of ewes. The negative sera were collected from three unvaccinated ewes and pooled in equal volume in one stock. The positive sera were collected from 3 vaccinated ewes, at 11 days after the second immunization with the SE vaccine, and pooled in similar procedure to that used for the negative control sera. The

coating used in the checkerboard was at two levels namely, 24 µg/100µl/well and 48 µg/100µl/well of the Immunolon 1-type of microtiter plates (Dynatech Laboratories, Virginia, USA).

The developing of ELISA compared the use of phosphate buffered saline (PBS) alone or PBS supplemented with 1 % Bovine Serum Albumin (BSA), as a diluent of the ewes' sera (1:100), and the conjugate (1:2000) included in the ELISA system. The replicates of the control sera were reacted on the micro-titer plates with each of the two protein-levels of coated SE antigens, in presence and absence of BSA supplementation to the buffer diluent. It is worth noting that the constants in the checkerboard were the coating buffer (carbonate/bicarbonate, pH 9.6) , conjugate dilution (Rabbit anti-complete sheep IgG, light and heavy chains, labeled with peroxidaze enzyme, Abcam, England), the substrate (ABTS 1-component containing 2,2-azino-di 3 ethyl- benzthiazoline-6-sulfonate),and the light wave length of 450 nm at which the developed color was read in the Biotek ELISA reader, model Elx 800, Biotek, USA.

C. Experimental Design

A total of 24 ewes, with an average age of 4 years, were divided into four treatments (T), with 6 ewes/ treatment. The T1 ewes were the negative controls, deprived of subcutaneous administration of the experimental SE vaccine and of oral administration of *Echinacea purpurea* (EP) dried roots. The T2 ewes were only treated with EP, while the T3 ewes were only vaccinated at D1 (initiation of the trial) and D10, and the T4 ewes were vaccinated at same days like the T3 ewes and administered the EP dried root tablets. The vaccine dose of 2 c.c was administered subcutaneously in the neck, while the EP treatment was a daily administration of

250 mg of dried roots of EP/tablet for a period of 45 days, effective the day of the first vaccination by the SE bacterin.

D. Blood Collection

The collection of the blood was performed from the jugular vein of all ewes at D1 (first SE vaccination day), D10 (booster SE vaccination day), D21 (11 days post booster), and D45 (35 days post the booster). The individual serum samples were collected from the clotted blood, and stored at -40ºC for analysis in duplicate on the optimized ELISA protocol.

E. Milk parameters

Individual milk volume was determined at days 1, 10, 21 and 45 using a graduated cylinder. Milk parameters namely: Fat, Solid Non-Fat, Protein, Density, and Lactose were determined using the Ekomilk Total Ultrasonic Milk Analyzer (Promishlena Str. 19 Stara Zagora, 6000, Bulgaria).

F. Statistical analysis

Covariate analysis was performed because the ewes were not of the same weight, milk production, and age. While the statistical analysis was done by one-way ANOVA, and significant differences are reported at $p \leq 0.05$; and the design was complete randomized since all of the ewes were grazing together. In order to check the significance between groups, Tukeys test was performed.

CHAPTER IV

RESULTS AND DISCUSSION

A. Checkerboard in optimization of the ELISA

The data of the checkerboard for optimization of ELISA system, targeting wider significant differences between the negative and the positive control sera, specific to SE antigens, are shown in Table 1. The absence of 1% BSA supplementation in PBS buffer of the diluent, at a lower coating level with 24 μg of SE antigens/well, resulted in similar and insignificant difference of means of negative (OD = 0.721)and positive replicates (OD = 0.868) of the control sera ($p>0.05$). The doubling of the SE antigen coating to 48 μg of SE/well, and in absence of BSA supplementation to the buffer diluent, created a significant difference in OD values obtained by negative (OD =0.602) and positive (OD = 0.822) control sera ($p<0.05$). This improvement in creating a difference between negative and positive control sera is most likely due to the filling of more surface area in the wells by the doubled amount of SE antigen, thus reducing the uncoated area, which most likely lead to less non-specific binding of antibodies in the control sera and of the secondary antibodies present in the conjugate (Xiao and Issacs, 2012).

A further improvement in differentiation between the negative and the positive control sera was obtained at a higher coating of 48μg SE antigens/well, and in the presence of 1% BSA-blocking protein in diluents of the control sera and the conjugate (Table 1). The 1 % BSA supplementation created a much wider and significant difference between the negative (OD =0.211) and positive (OD = 0.747) control sera ($p<0.05$).The 1 % BSA seems to block the

surface area of the wells that are left uncoated with the SE antigen, thus preventing significantly the nonspecific binding of antibodies in the control sera and those in the conjugate (Xiao and Issacs, 2012).

The results of the checkerboard shown in Table 1 lead to final optimized protocol of the developed ELISA, using 48μg of SE protein/well/100 μl of the carbonate/ bicarbonate coating buffer (pH 9.6), dilution of ewes sera at 1:100, and conjugate dilutionat1:2000, with supplementation of 1 % BSA in the PBS used in diluting the ewes sera and the conjugate.

B. Ewes acquired immunity to SE vaccine and/or EP

The data resulting from the application of the optimized ELISA for quantitating the acquired humoral primary and secondary seroconversion in the four different treatments of ewes, following two vaccinations by the experimental SE bacterin and/or *Echinacea Purpurea* (EP) treatment, is shown in Table 2. The ewes in treatments T1 and T2 did not seroconvert to higher mean optical densities, at any of the sampling times of D10, D21, and D45, compared to the original background mean optical densities at D1. This confirms the absence of exposure of ewes in T1 and T2 treatments to SE antigens, due to deprivation from SE vaccination, and the absence of SE contamination in the experimental facility. In addition, the daily treatment of the ewes in T2 with EP did not result in any plasma cell formation from innate clone of mature B-cells that potentially carry the IgM and IgD isotypes on their surfaces, with idiotypic specificity to SE antigens. The EP seems to boost the proliferation of B-cells in the presence of certain mitogen or antigen, as described previously (Barbour et al., 1993; Skopinska-Rozewske et al., 2011; Rehman et al., 1999).

The administration of only the developed SE bacterin to ewes of T3 treatment at D1 and D10 did result in significant acquired humoral primary response, detected at D10 after the first vaccination (mean OD value = 0.658) compared to mean OD value of 0.273 at D1 ($p<0.05$) . Unfortunately, the boosting at D10 in T3 ewes did not result in significant seroconversion at D21 (mean OD value = 0.698) compared to the titer at boosting time of D10 (mean OD value = 0.658) ($p>0.05$). This indicates that the boosting with this developed vaccine was not able to create a class switch in the IgM-producing plasma cells formed by the first vaccination to IgG-producing plasma cells, which is most likely due to failure of T-cell activation that is needed in cooperating with the B-cell for producing a class switch (Mitchison, 2004).

Regarding the ewes of T4 treatment, they showed a success in induction of a significant primary and secondary humoral immune responses by the same vaccine batch that was administrated to T3 ewes. The additional treatment applied to T4 ewes of daily administration of EP tablets (250 mg dried root/tablet/ewe) between D1 to D45, seems to be the only factor that helped in obtaining a significant seroconversion of the secondary response. The primary response detected at D10 (mean OD = 0.561) following the first vaccination at D1 (mean OD = 0.287) of T4 ewes was significant ($p<0.05$). The booster given at D10 to these ewes led to a secondary response with significant seroconversion (mean OD value = 0.701) compared to mean OD value at D10 (0.561) ($p<0.05$).The EP seems to help in activation of T-cells leading to efficient cooperation with B-cells to create such a statistically significant seroconversion.

This is the first data in literature reporting the synergism between the developed SE bacterin and the EP treatment in ewes for creation of significance in both the primary and secondary humoral immune responses. This data is in agreement with previous works done on different animal

models, including mice (Freier et al., 2003), rats (Zhai et al., 2006) and humans (Brush et al., 2006).

It is worth noting that the period between D21 and D45 is 24 days, a period of normal decay in the acquired SE-specific antibodies of the ewes, due to their well-documented half-life of 12-17 days (Watson, 1992). This decline was noted at D45 in T3 ewes (OD =0.572) and T4 ewes (OD =0.639) compared to the respective secondary response present at D21 (mean OD of 0.698 and 0.701) ($p < 0.05$).However the decline in mean OD value at D45 compared to that of D21 in T3 ewes was 18.1 %, while the decline in mean OD values of T4 ewes was equivalent to 8.8 %. However, the mean OD value at D45 of T4 ewes (0.639) was still significantly higher than that of the primary response obtained at D10 (0.561)($p<0.05$), while the T3 ewes had a significantly lower mean OD valve at D45 (0.572) compared to that of the primary response at D10 (0.658)($p<0.05$). These results indicate that the EP treatment in T4 ewes can help to reduce the effect of half-life of acquired SE-specific antibody through the 35 days following the booster that was administered at D10.

In conclusion, the optimization of the ELISA by the checkerboard strategy was able to widen significantly the difference in the mean OD values between the negative and positive control sera specific to SE antigens. In addition, the application of the optimized ELISA helped in documenting the differences in seroconversion of ewes that were differently treated, uncovering the benefit of *Echinacea purpurea* treatment in overcoming the failure of the experimental SE bacterin to induce a significant seroconversion of the secondary immune response, and in helping the reduction in the decay of acquired SE-specific antibodies by time.

Table1. Checkerboard for optimization of ELISA system to detect wider significant differences between negative and positive control sera specific to SE antigens

Control Sera	Mean[1] optical density values at different levels of coated SE antigen in presence and absence of BSA[2]			
	24µg SE antigens/100µl of coating buffer/well		48µg SE antigens/100µl of coating buffer/well	
	BSA		BSA	
	presence	absence	presence	absence
Negative[3]	0.2045 [a1]	0.7207 [a2]	0.211[a1]	0.602 [a2]
Positive[4]	0.623[b1]	0.868 a[2]	0.747[b1]	0.822[b1]
SEM[5]	0.082	0.034	0.064	0.025

[1]Mean of six replicates of each of negative and positive control sera

[2]BSA = Bovine Serum Albumin added at 1 % level to PBS buffer used in diluting the control sera and the conjugate

[3]Negative control serum is pooled in equal volume from 6 ewes of T2 treatment deprived of SE vaccination, and collected at day 21 (D21).Dilution used is 1:100

[4]Positive control serum is pooled in equal volume from sera of 6 ewes of T4 treatment that were SE-vaccinated and treated with *Echinacea purpurea*, and collected at day 21 (11 days post the booster).

[5]SEM = Standard Error of Mean

[1,2]Twomean in a row, under the coating by same SE antigen level, followed by different Arabic numerical superscripts are significantly different at $p<0.05$.

[a-b]Means in a column, followed by different Alphabet super scripts are significantly different at $p<0.05$.

Table 2.The application of the optimized ELISA[1] in quantitating the acquired humoral primary and secondary seroconversion in the four different treatments of ewes following two vaccinations[2] by the developed SE bacterin and/or *Echinacea Purpurea* (EP)[3]

Treatments[4]	Means of acquired humoral immunity specific to SE (OD value) at different sampling days (D)				
	D1	D10	D21	D45	SEM[6]
T1	0.2358 [a1,2]	0.2641[a2]	0.281[a2]	0.2137[a1]	0.012
T2	0.3037[a1]	0.2707[a1]	0.2583[a1]	0.2266[a2]	0.013
T3	0.2727 [a1]	0.6578[b2]	0.6985 [b2]	0.5718[b3]	0.015
T4	0.2868 [a1]	0.561 [b2]	0.7007[b3]	0.6385[b4]	0.018
SEM[7]	0.026	0.038	0.024	0.028	

[1]Optimized ELISA is constructed of 48 µg SE antigens/100µl coating buffer/well, respective dilution of ewes sera and conjugate at 1:100 and 1:2000, and supplementation of PBS buffer diluent of ewes sera and conjugate with 1% bovine serum albumin

[2]SE vaccination of ewes at D1 and D10, in 2 ml/dose, administered subcutaneously in the neck of ewes in T3 and T4 treatments.

[3]EP daily treatment to ewes of T4 treatment, effective D1 and up to D45, administered intra-esophageally in a dose of 250 mg of dried root/tablet/ewe.

[4]T1 has control negative ewes, non-vaccinated and non-treated with EP, T2 has ewes deprived of vaccination and treated with EP, T3 has ewes administrated SE vaccine at D1 and D10, and

deprived of EP treatment, T4 has ewes administrated SE vaccine at D1 and D10, and treated with EP.

[5]Means of OD = Means of Optical Density values of the sera of 6 ewes/treatment, with each serum run in duplicate.

[6]SEM = Standard Error of Means in a row.

[7]SEM = Standard Error of Means in a column.

[1-4]Mean in a row followed by different Arabic numerical superscripts are significantly different at $p<0.05$.

[a-b]Mean in a column followed by different Alphabet superscripts are significantly different at $p<0.05$.

C. Milk Parameters

Table 3 shows the average milk decrease%, SNF, protein, density, lactose, and fat percentage in milk of the experimental ewes during the whole experiment.

Table 3. Milk parameters of the experimental groups

Trt	Control	Herb	Vaccine	Herb & Vaccine	
Group[1]	T1	T2	T3	T4	SEM
SNF (%)	10.85	11.133	10.713	11.456	0.15
Protein (%)	5.575	5.857	5.486	5.808	0.074
Density (g/cm^3)	1.039	1.043	1.039	1.041	0.00076
Lactose (%)	4.451	4.949	4.336	4.762	0.104
Conductivity (mS/cm)	4.129	3.588	3.587	3.779	0.045
Fat (%)	4.72	5.435	5.615	5.868	0.24
Milk decrease (%)	43.71	45.43	51.59	44.66	2.56

[1]T1 has control negative ewes, non-vaccinated and non-treated with EP, T2 has ewes deprived of vaccination and treated with EP, T3 has ewes administrated SE vaccine at D1 and D10, and deprived of EP treatment, T4 has ewes administrated SE vaccine at D1 and D10, and treated with EP.

There was no significant difference among various milk parameters for different experimental groups. However the results of groups 2 and 4 were the highest in SNF, protein, density, and lactose compared to those of groups 1 and 3. This could be explained by the fact that ewes of groups 2 and 4 were administered the herbal extract but at values lower than those reported in literature. Kholif et al, (2004), Allam et al. (1999) and Al-Shaikh et al. (1999) had significant improvements in milk parameters as a result of using higher amount of fenugreek ranging between 1000 and 1200 g / day.

Kholif and Shewy (2004) found that fenugreek seed supplementation in 27 Baladi goats increased milk fat and protein yields significantly. However, total solids, milk proteins, fat, ash, lactose and SNF were not affected, as found out in our study.

Allam et al. (1999) studied the effect of fenugreek seed at the level of 500 mg/kg LBW in Zaraibi goat and found an increase in average daily milk yield and fat corrected milk. However, total solids, CP and lactose percentages were alike.

Al-Shaikh et al. (1999) supplemented 0, 25 and 50 per cent fenugreek seeds in the concentrate mixtures in the diet of goats and detected higher milk yield and fat per cent at 25% levels.

CHAPTER V

CONCLUSION AND RECOMMENDATIONS

The optimization of the ELISA by the checkerboard strategy was able to widen significantly the difference in the mean OD values between the negative and positive control sera specific to SE antigens. The application of the optimized ELISA helped in documenting the differences in seroconversion of ewes that were differently treated, uncovering the benefit of herbal treatment in overcoming the failure of the experimental SE vaccine to induce a significant seroconversion of the secondary immune response, and in helping the reduction in the decay of acquired SE-specific antibodies by time.

Regarding the milk parameters, the utilization of fenugreek at the level of 610 mg/day did not significantly increase milk yield, or affect SNF, fat, proteins, conductivity, lactose and density of the milk.

It is recommended to combine the inclusion of EP extract with the use of vaccines to improve the immunity and enhance the sera titers in Awassi ewes. This combination seems to be beneficial for disease control in Awassi ewes, leading to the reduction of antibiotics use in small ruminants farming.

Fenugreek is a galactagogue that improves milk parameters; which is a better alternative than using hormones; however it should be included in the diet in higher rates. This will be the target of future research for the improvement of milk production and milk parameters in Awassi ewes.

Future research is needed to elucidate the effect of the two plants separately and independently as this was not possible under the current experimental design.

BIBLIOGRAPHY

Liu, F., Tang, W.J., Cheng, A.C., Wang, M.S., Zhao, T.T., Han, Q.L. and Gao, L.Q. (2011). Preparation of anti-GPV yolk IgG and development of an indirect-ELISA for detection of goose parvovirus infection in goose. Chin Vet Sci 41, 601–606.

Shang, X.-Z., Zhang, Y.-W., Wang, Z.-P., Ju, H.-Y., Ma, B. and Wang, J.-W. (2010). Establishment of an indirect ELISA for detection of antibodies against NS2 protein of Gosling plague. Chin J Prev Vet Med 32, 595–598. against avian hepatitis E virus. Journal of Virological Methods, 187, 32– 36.

Balamurugan, V., Singh, R.P., Saravanan, P., Sen, A., Sarkar, J., Sahay, B., Rasool, T.J., Singh, R.K. (2007). Development of an Indirect ELISA for the Detection of Antibodies against Peste-des-petits-ruminants Virus in Small Ruminants. Veterinary Research Communications, 31, 355–364.

Bang, J., Shukla, S., Kim, Y.H., Kim, M. (2012). Development of indirect competitive ELISA for the detection of Salmonella Typhimurium. Romanian Biotechnological Letters, 17(2), 7194-7204.

Dalby, T., Strid, M. A., Beyer, N. H., Blom, J., Mølbak, K. &Krogfelt, K. A. (2005). Rapid decay of Salmonella flagella antibodies during humangastroenteritis: a follow up study. J Microbiol Methods 62, 233–243.

Dufour-Zavala, L., Swayne, D.E., Glisson, J.R., Pearson, J.E., Reed, W.M., Jackwood, M.W., Woolcock, P.R. (2008). A Laboratory Manual for the Isolation and Identification of Avian Pathogens. Kennett Square, PA: American Association of Avian Pathologists. 249 pp.

Fan, J. H. Zuo, Y. Z., Yang Z., Pei L. H. (2012) The development of an indirect ELISA for the detection of antibodies to goose parvovirus in blood serum. Letters in Applied Microbiology, 57, 26-32.

Gast, R. K., Nasir, M. S., Jolley, M. E., Holt, P. S. & Stone, H. D. (2002). Serologic detection of experimental *Salmonella enteritidis* infections in laying hens by fluorescence polarization and enzyme immunoassay. Avian Dis 46, 137–142.

House, D., Chinh, N. T., Diep, T. S., Parry, C. M., Wain, J., Dougan, G., White, N. J., Hien, T. T. & Farrar, J. J. (2005). Use of paired serum samples for serodiagnosis of typhoid fever. J ClinMicrobiol 43, 4889–4890.

Mahgoub, E. (2014) The Development and Application of an Indirect ELISA Test for the Detection of Chicken Anaemia Virus (CAV) by VP1 in Chicken Flock Serum Open Journal of Genetics, 4, 316-331.

Pererat, K., Murray, A. (2009) Development of an indirect ELISA for the detection of serum IgG antibodies against region IV of phase 1 flagellin of Salmonella entericaserovar Brandenburg in sheep. Journal of Medical Microbiology, 58, 1576–1581.

S. KIM, Salmonella serovars from foodborne and waterborne diseases in Korea, 1998-2007: total isolates decreasing versus rare serovars emerging. J. Korean Med. Sci., 25, 1693-1699 (2010).

Saif, Y.M., Barnes, H.J., Fadly, A.M., Glisson, J.R., McDougald, L.R., Swayne, D.E. (2008). Diseases of Poultry. Ames, Iowa: Iowa State University Press; 1352 pp.

Veling, J., van Zijderveld, F. G., van Zijderveld-van Bemmel, A. M., Barkema, H. W. &Schukken, Y. H. (2000). Evaluation of three newly developed enzyme-linked immunosorbent assays and two agglutination tests for detecting Salmonella enterica subsp. entericaserovar Dublin infections in dairy cattle. J ClinMicrobiol 38, 4402–4407.

Wang, G., Ding, J., Hu, S., YangX. (2012). Development and preliminary application of an indirect ELISA for detecting antibodies against Avian Influenza Virus. Research in Veterinary Science, 93, 710–715.

Zhao, Q., Sun, Y., Zhao, J., Hu, S., Zhao, F., Chen, F., Clavijo, A., Zhou, E., Xiao, Y. (2013).Development and application of an indirect ELISA for detection of antibodies.

Zoubiane, G. (1995). Postpartum fertility and immune response in lactating Awassi and Finn X Texel X Awassi ewes. MS Thesis. Department of Animal Sciences, American University of Beirut. 122 pp.

Kumar, S; Gupta P; Sharma S; Kumar D (2011). "A review on immunostimulatory plants". Journal of Chinese Integrative Medicine9 (2): 117–128.

Akhtar, M.S., Z. Nasir, A.R. Abid, (2003): Effect of feeding powdered Nigella sativa L. seedson poultry egg production and their suitability for human consumption. VeterinarskiArhiv.73(3), 181-190.

Nasir, Z., M.A. Grashorn, (2006): Use of Black cumin (Nigella sativa) as alternative to antibiotics in poultry diets. 9th TagungSchweine-und Geflügelernährung, Halle (Saale), Germany. pp 210-213.

Nasir, Z., M.A. Grashorn, (2009a): Echinacea: A potential feed and water additive in poultry and swine production. Arch. Geflügelk. 73(3), (In press).

Barrett, B., 2003: Medicinal properties of Echinacea: a critical review. Phytomed. 10, 66-86.

Zhai, Z., L. Yi, L. Wu, D.S. Senchina, E.S. Wurtele, P.A. Murphy, M.L. Kohut, J.E. Cunnik. (2007). Enhancement of innate and adaptive immune functions by multiple Echinacea species. J. Med. Food 10(3), 423-434

Nasir, Z., M.A. Grashorn. (2009). Echinacea: A potential feed and water additive in poultry and swine production. Arch. Geflügelk. 73(3), (In press).

Woelkart, K., R. Bauer. (2007). The role of alkamides as an active principle of Echinacea. Planta Med. 73, 615-623.

Rininger, J.A., S. KickneR, P. Chigurupati, A. MClean, Z. Franck (2000). Immunopharmacological activity of Echinacea preparations following simulated digestion on murine macrophages and human peripheral blood mononuclear cells. J. Leukocyte Biol. 68,503-510.

Doaa, F. T.; El-Saied, U. M.; Sallam, A. A.; El-Baz, A. M.; Hussein, A. M. (2009). Effect of using Echinacea extract as immuno-stimulating additive on milk yield traits, immunity and udder health of Zaraibi goats. Egyptian Journal of Sheep and Goat Sciences, 4 (2): 33-53.

Sgorlon S, Colitti M, Asquini E, Ferrarini A, Pallavicini A, Stefanon B. (2012). Administration of botanicals with the diet regulates gene expression in peripheral blood cells of Sarda sheep during ACTH challenge. Domestic animal endocrinology 43 (3) : 213-26.

Reklewska, B, Bernatowicz, E, Ryniewicz, Z, Rua Pinto, R and Zdziarski, K (2004). Preliminary observations on the Echinacea-induced lactoferrin production in goat milk. Anim. Sci. Papers and Reports, 22 (1): 17-25.

Hrivniakova L, Schmid D, Luckner-Hornischer A, Lassnig H, Kornschober C, Angermayer J, et al. Salmonellosis outbreak due to *Salmonella enteritidis* phage type 14b resistant to nalidixic acid, Austria, September (2010). Euro surveillance: bulletin européen sur les maladies transmissibles = European communicable disease bulletin. 2011;16(34).

Centers for Disease Control and Prevention (2010).

Peters TM, Berghold C, Brown D, Coia J, Dionisi AM, Echeita A, et al. (2007). Relationship of pulsed-field profiles with key phage types of Salmonella enterica serotype Enteritidis in Europe: results of an international multi-centre study. Epidemiology and infection. 135(8):1274-81.

European Food Safety Authority and European Centre for Disease Prevention and Control. The European Union Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Food-borne Outbreaks in 2012. EFSA Journal 2014;12(2):3547 doi:10.2903/j.efsa.2014.3547.

EFSA BIOHAZ Panel (EFSA Panel on Biological Hazards). Scientific Opinion on the public health risks of table eggs due to deterioration and development of pathogens. EFSA Journal. 2014;12(7):3782.

JanMohamed K, Zenner D, Little C, Lane C, Wain J, Charlett A, et al. National outbreak of *Salmonella Enteritidis* phage type 14b in England, September to December 2009: case-control study. Euro surveillance: bulletin européen sur les maladies transmissibles = European communicable disease bulletin. 2011;16(15).

Styles T, Phan Q, Rabatsky-Ehr T, Applewhite C, Sosa L, Cartter M.(2013). Salmonella enterica serotype enteritidis outbreak at a long-term care facility, Connecticut, 2012. Conn Med., 77(10):587-90.

Banchereau J, et al. (2000). Immunobiology of dendritic cells. Annu Rev Immunol.

Benson JM, et al. (2010). *Echinacea purpurea* extracts modulate murine dendritic cell fate and function. Food ChemToxicol.

Britton, ER, DA Todd , M Leyte-Lugo , T Gulledge LF Grubbs M Oberhofer , SH Faeth SM Laster NB Cech Unraveling immunomodulatory activities of complex *Echinacea purpurea* extracts.

Dapas B, Dall'Acqua S, Bulla R, Agostinis C, Perissutti B, Invernizzi S, Grassi G, Voinovich D Dong GC, et al. (2009). Blocking effect of an immuno-suppressive agent, cynarin, on CD28 of T-cell receptor. Pharm Res.

Fonseca FN, Papanicolaou G, Lin H, Lau CB, Kennelly EJ, Cassileth BR, (2013). Cunningham-Rundles S. *Echinacea purpurea* (L.) Moench modulates human T-cell cytokine response. IntImmunopharmacol. 2014 Mar;19(1):94-102. doi: 10.1016/j.intimp. Epub 2014 Jan 13.

Gertsch J, et al. (2004). Echinacea alkylamides modulate TNF-alpha gene expression via cannabinoid receptor CB2 and multiple signal transduction pathways. FEBS Lett.

Goel V, et al. (2002). Alkylamides of *Echinacea purpurea* stimulate alveolar macrophage function in normal rats. IntImmunopharmacol.

Immunomodulation mediated by a herbal syrup containing a standardized Echinacea root extract: A pilot study in healthy human subjects on cytokine gene expression.Phytomedicine. 2014 Sep 25;21(11):1406-10. doi: 10.1016/j.phymed.2014.04.034. Epub 2014 May 28.

Kapai NA, et al. (2011). Selective cytokine-inducing effects of low dose Echinacea. Bull ExpBiol Med.

Mishima S, et al. (2004). Antioxidant and immuno-enhancing effects of *Echinacea purpurea*. Biol Pharm Bull.

Morazzoni P, et al. (2005). In vitro and in vivo immune stimulating effects of a new standardized Echinacea angustifolia root extract (Polinacea). Fitoterapia.

Nadja B Cech, Katrina Tutor, Bethany A DotyKevin SpelmanMasa SasagawaGregory M RanerCynthia A Wenner Liver enzyme-mediated oxidation of *Echinacea purpurea* alkylamides: production of novel metabolites and changes in immunomodulatory activity.

Pertwee RG. (2006). The pharmacology of cannabinoid receptors and their ligands: an overview. Int J Obes (Lond).

Planta Med 2014 DOI: 10.1055/s-0034-1382726

Planta Medica (Impact Factor: 2.35). 01/2007; 72(15):1372-7. DOI: 10.1055/s-2006-951718\

Rehman J, et al. (1999). Increased production of antigen-specific immunoglobulins G and M following in vivo treatment with the medicinal plants Echinacea angustifolia and Hydrastiscanadensis. Immunol Lett.

Ritchie MR, et al. (2011). Effects of Echinaforce® treatment on ex vivo-stimulated blood cells. Phytomedicine.

Sasagawa M, et al. (2006). Echinacea alkylamides inhibit interleukin-2 production by Jurkat T cells. IntImmunopharmacol.

Schwarz E, et al. (2005). Effect of oral administration of freshly pressed juice of *Echinacea purpurea* on the number of various subpopulations of B- and T-lymphocytes in healthy volunteers: results of a double-blind, placebo-controlled cross-over study. Phytomedicine.

Senchina DS, et al. (2011). Phytochemical and immunomodulatory properties of an Echinacea laevigata (Asteraceae) tincture. J Altern Complement Med.

Skopińska-Rózewska E, et al. (2011). Dose-dependent in vivo effect of Rhodiola and Echinacea on the mitogen-induced lymphocyte proliferation in mice. Pol J Vet Sci.

Stevenson LM, et al. (2005). Modulation of macrophage immune responses by Echinacea. Molecules.

Sullivan AM, et al. (2008). Echinacea-induced macrophage activation. ImmunopharmacolImmunotoxicol.

Wang CY, et al. (2008). Genomics and proteomics of immune modulatory effects of a butanol fraction of echinaceapurpurea in human dendritic cells. BMC Genomics.

Wang CY, et al. (2006). Modulatory effects of *Echinacea purpurea* extracts on human dendritic cells: a cell- and gene-based study. Genomics.

Yu D, et al. (2013). Anti-inflammatory effects of essential oil in *Echinacea purpurea* L. Pak J Pharm Sci.

Zhai Z, et al. (2007). Enhancement of innate and adaptive immune functions by multiple Echinacea species. J Med Food.

Santos SB, Kropinski AM, Ceyssens P.J, Ferreira E.C, and Azeredo J. (2011). Genomic and proteomic characterization of the broad-host-range Salmonella phage PVP-SE1:Creation of a new phage genus. J Virol ;85:11265-73

Barbour EK, Jurdi L.H, Talhouk R, Qatanani M, and Spasojevic R. (1999). Emergence of *Salmonella enteritidis* outbreaks in broiler chickens in Lebanon. Epidemiological markers and competitive exclusion control. Rev Sci Tech;18:710-718.

United States Environmental Protection Agency. (2013). Literature review of contaminants in livestock and poultry manure and implications for water quality; Pp.1-137

Chen Z, and Jiang X. (2014). Microbiological safety of chicken litter or chicken litter-based organic fertilizers: A review. Agriculture;4:1-29.

Davier RH, Breslin M. (2003). Persistence of *Salmonella Enteritidis* phage Type 4 in the environment and arthropod vectors on an empty free range chicken farm. Environ Microb;5:79-84.

Nicholson FA, Groves SJ, Chambers BJ. (2005). Pathogen survival during livestock manure storage and following land application. BioresourceTechnol;96:135-143.

Peters TM, Berghold C, Brown D, Coia J, Dionis AM, Echeita A, Fisher IST, Gato AC. (2007). Relationship of pulsed-field profiles with key phage type of Salmonella Enterica serotype Enteritidis in Europe: results of an international multi-centre study. Epidemiol Infect;135:1274-1281.

Lewis RD, Meyer HH, Gradin JL, Smith AW. (1989). Effectiveness of vaccination in controlling ovine foot rot. J AnimSci;67:1160-1166.

Wilsomore AJ, Wilsmore BC, Dagnall GJR, Izzard RA, Woodland RM, Dawson M, and Venables C. (1990). Clinical and immunological responses of ewes following vaccination with an experimental formalin inactivated Chlamydia psittaciovis vaccine and subsequent challenge with the live organism during pregnancy . Br Vet J;146:341-348.

Barbour E.K, Hamadeh S.K, Zoubiane G, Talhouk R, Hilan C. (1996). An enzyme-linked-immunosorbent-assay for evaluation of an experimental Salmonella Typhimurium vaccine in 2 breeds of ewes. Small Ruminant Research;21:239-244.

Barbour EK, Wayne WM, Nabbut NH, Poss PE, Brinton MK. (1993). Evaluation of bacterins containing three predominant phase types of *Salmonella enteritidis* for prevention of infection in egg laying chickens. Am J Vet Res;54:1306-1309

Singh BR. (2009). Salmonella vaccines of animals and birds and their future prespective. TOVACJ;2:100-112.

Kassaify ZG, Banat G, Baydoun E, Barbour EK. (2011). Quantitative assessment of fimbriae-specific serum and egg yolk antibodies induced in chicken layers by a newly developed live *Salmonella Enteritidis* vaccine and relationship to infection. Intern J Appl Res Vet Med;9(3):254-264.

Qiu YHL, Cui BA, Zhang HY, Rong XF, Wang DY, Wong YG. (2007). Immunopotentiating effects of four Chinese herbal polysaccharides administered at vaccination in chickens. PoultSci;86(12):2530-2535

Bagherwal P, Machawal L. (2013). Immune response modulation to DPT (Diphtheria, Pertussia, Tetanus) Vaccine by NDL from Calotropisprocera. IJPBS;4(2):852-859.

Barbour EK, Shaib H, Azhar E, Kumosani T, Iyer A, Harakeh A, and Bragg RR. (2013). Modulation by essential oil of vaccine response and production improvement in chicken challenged with velogenic Newcastle disease virus. J App Microbiol;115:1278-1286.

Skopinska-Rozewske E, Sokolnicka I, Siwicki A, Stankiewicz W, Dabrowski M, Buchwald W, Krajewska-patan A, Mielcarek S, Mscisz A, Furmanowa M. (2011). Dose-dependent in vivo effect of Rhodiola and Echinacea on the mitogen-enduced lymphocyte proliferation in mice. Pol J Vet Sci;14(2):265-272.

Rehman J, Dillow JM, Carter SM, Chou J, Le B, Maisel AS. (1999). Increased production of antigen-specific immunoglobulins G and M following in vivo treatment with the medicinal plants Echinacea angustifolia and Hydrastiscanadensis. Immunol Lett, 68(2-3):391-395.

Balamurugon V, Singh R.P, Saravanan P, Sen A, SarkarJ, Sahay B, Rasool T.J, Singh R.K. (2007). Development of an indirect ELISA for the detection of antibodies against pest-des-petits-ruminants virus in small ruminants. Vet Res Commun, 31:355-364.

Fan JH, Zno YZ, Yang Z, Pei LH. (2012). The development of an indirect ELISA for the detection of antibodies to goose parvovirus in blood serum. Lett ApplMicrobiol, 57:26-312.

Shang XZ, Zhang YW, Wang ZP, Ju HY, Ma B, Wang JW. (2010). Establishment of an indirect ELISA for detection of antibodies against NS2 protein of gosling plague. Chin J Prem Vet Med, 32:595-598.

Mahgoub E. (2014). The development and application of an indirect ELISA test for the detection of Chicken Anemia Virus (CAV) by VP1 in chicken flock serum. Open J Gen, 4:316-331.

Powell. (1995). Correlation of change in phage type with pulsed field profile and 16S rrn profile in Salmonella enteritidis phage types 4, 7, and 9a. Epidemiol Infect, 114(3): 403-11.

Xiao Y, and Issacs SN. (2012). Enzyme-linked immunosorbent assay (ELISA) and blocking with bovine serum albumin (BSA). J Immunol Methods;384: 148-151.

Mitchison NA. (2004). T-cell-B-cell cooperation. Nature reviews immunology;4:308-312.

Freier DO, Wright K, Klein K, Voll D, Dabiri K, Cosulich K, George R. (2003). Immunopharmacol. Immunotoxicol;25:551-560.

Zhai Z, Liu Yi Wu L, Senchina DS, Wurtele ES, Murphy PA, Kohut ML, Cunnick JE. (2007). Enhancement of innate and adaptive immune functions by multiple Echinacea species. J Medicinal Food, 10:423-434.

Brush J, Mendenhall E, Guggenheim A, Chan T, Connelly E, Soumyanath A, Buresh R, Barrett R, and Zwickey H. (2006). The effect of *Echinacea purpurea* ,Astragalusmembranaceus and Glycyrrhizaglabra on CD 69 expression and immune cell activation. Phytother Res, 20:687-695.

Watson DL. (1992). Biological half-life of ovine antibody in neonatal lambs and adult sheep following passive immunization. Vet Immunopathol, 30:221-232.

M. A. Al-Shaikh , S. I. Al-Mufarrej& H. H. Mogawer (1999) Effect of Fenugreek Seeds (Trigonellafoenumgraecum L) on Lactational Performance of Dairy Goat, Journal of Applied Animal Research, 16:2, 177-183, DOI: 10.1080/09712119.1999.9706279.

D. Taskin, U. Halil, O. Selda, and K. Hasan (2016) Effect of ground fenugreek seeds (Trigonellafoenum-graecum) on feed consumption and milk performance in Anatolian water buffaloes, Arch. Anim. Breed., 59, 345.

S. Shabana, S. Michelle , K. Sushant ,D. Nancy , A. Michael , and K. Saeed (2011) Fenugreek: a naturally occurring edible spice as an anticancer agent, Cancer BiolTher, 8(3): 272–278.

Rejil, M. C. (2005). *Mitigation of methane emission from ruminants by dietary supplementation of fenugreek seeds* (Doctoral dissertation, NDRI, Karnal).

Jain, P., Mohini, M., Singhal, K. K., &Tyagi, A. K. (2011). Effect of herbal mixture supplementation on methane emission and milk production in cattle. *Indian Journal of Animal Nutrition*, *28*(4), 377-384.

Bin-Hafeez, B., Haque, R., Parvez, S., Pandey, S., Sayeed, I., & Raisuddin, S. (2003). Immunomodulatory effects of fenugreek (Trigonella foenum graecum L.) extract in mice. *International immunopharmacology*, *3*(2), 257-265.

Anuradha, C. V., & Ravikumar, P. (2001). Restoration on tissue antioxidants by fenugreek seeds (Trigonella Foenum Graecum) in alloxan-diabetic rats. *Indian Journal of Physiology and Pharmacology*, *45*(4), 408-420.

Pandian, R. S., Anuradha, C. V., & Viswanathan, P. (2002). Gastroprotective effect of fenugreek seeds (Trigonella foenum graecum) on experimental gastric ulcer in rats. *Journal of ethnopharmacology*, *81*(3), 393-397.