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Effects of Dietary Antioxidants Supplementation on Cellular Immune Response and Evaluation of Their Antimicrobial Activity Against Some Enteric Pathogens in Goats

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Abstract: The present study aimed to investigate the effects of the use of natural antioxidant such as *Nigella sativa* or black cumin seeds (BCS) and organic multi-nutrient antioxidants, as dietary supplements on cellular immune status and evaluate their antimicrobial effects on some enteric pathogens (*E. coli* and *C. perfringens*) in goats. We also aimed to detect certain virulence genes of these pathogens using Multiplex PCR. Fifteen, 4-6 months old Baladi goat kids were divided into three equal groups (n=5). Group A was kept as control and fed the basal diet, group B received basal diet supplemented with crushed BCS at ratio of 2% and group C received basal diet supplemented with the organic multi-nutrient antioxidants; zinc methionine (Zn-Met.) and vitamin E with selenium enriched yeast (Vit E\Se) at levels of 2g\kg of diet. All treatments extended for 120 days. Heparinized blood samples were aseptically collected from all goats at day 0 and once at the end of experimental period for immune cell function studies. The cellular immune responses of experimental animals were assessed by lymphocyte proliferation using XTT kit and phagocytic % & phagocytic index using *candida albicans*. Fecal samples were collected at day 0 and subsequently at 30 days intervals up to 120 days of experimental period for bacteriological examination and total bacterial counts. Multiplex PCR was carried out for detection of virulent genes F41 and K99 genes for *E. coli* and alpha, beta and epsilon toxin genes for *C. perfringens*. The obtained results revealed a significant elevation of lymphocyte and phagocytic activities in groups B and C received antioxidants compared with goats in group A fed the basal diet. Moreover, BCS showed antimicrobial effect against some enteric pathogens by inhibition of the intestinal bacterial counts of *E. coli* and *C. perfringens*. Detection of some certain virulent genes for the studied pathogens showed positive results to "K99" gene for *E. coli* and alpha & epsilon toxin genes for *C. perfringens type D.* In conclusion, dietary supplementation with either *N. sativa* or multi- nutrient antioxidants (Zn-Met. and Vit E\Se) can be used to provide potent immune cell function in goats. Moreover, *N. sativa* had proved to have antibacterial effect against some enteric pathogens.

Key words: Antioxidants · Vit E. Selenium · Zinc · *Nigella sativa* · Cellular Immunity · *E. Coli* · *Clostridia perfringens* · Goat

INTRODUCTION

Antioxidants have been defined as molecules that prevent cell damage against free radicals and are critical for maintaining optimum health in both animals and humans. In all living systems, cells require adequate levels of antioxidant defenses in order to avoid the harmful effect of an excessive production of reactive oxygen species (ROS) and to prevent damage to the immune cells [1]. Spears and Weiss [2] stated that these cells contain a high amount of polyunsaturated fatty acids in their cell membranes and are very susceptible to “peroxidation”.

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Certain nutrients such as vitamin E, Se and Zn are common antioxidants normally included in animal diet that improve different immune functions exhibiting an important protective role in infections caused by bacteria, viruses or parasites [3-5]. As a result, dietary antioxidants have been related to modulate the host susceptibility or resistance to infectious pathogens [1]. Combinations of antioxidants may be more effective than larger quantity of any single one [6, 7]. Interestingly, a high concentration of such nutrients in the diet above predicted requirements prevented protein and lipid oxidation and improve immune functions in ruminants [4, 8].

Vitamin E along with Se form a vital part of biological antioxidant system [9]. They are involved in the protection of biological membranes against lipid peroxidation. Selenium as an essential component of glutathione peroxidase (GPx), acts to destroy peroxides before they attack cell membranes [10], while vitamin E was reported to act within the membrane to prevent the formation of fatty acid hydro peroxides [11]. Likewise, Zinc is effective as antioxidant and has a catalytic, coactive, or structural role in a wide variety of enzymes that regulate many physiological processes including metabolism, growth and immune function and is also involved in gene expression at the transcription level [12, 13]. In the majority of investigations it was shown that Vitamin E, Se and Zn supplementation improves immune function in ruminants [7, 14-16].

Nowadays, there is an increased demand for using plants in therapy "back to nature" instead of using synthetic drugs which may have adverse effects. One of the alternatives used as feed additives is *Nigella sativa (N. sativa)* [17, 18]. The seed of *N. sativa* has been reported to have many biological properties including antibacterial [19], antiviral [20] and anti-parasitic [21]. The oil fraction of *N. sativa* contains thymoquinone, which has immuno-potentiating activities as well as antioxidative effect [22, 23]. Besides, *N. sativa* seeds provide relatively high amounts of some essential nutrients such as carbohydrates, fats, vitamins, mineral elements and proteins including eight of the nine essential amino acids that improve natural immune system activity [24, 25].

The main objective of our study was to evaluate dietary supplementation of different forms of antioxidants (*N. sativa vs* multi nutrient- antioxidants; Zn-Met and Vit. E with Se enriched yeast) on cellular immune status of goats and investigate their antimicrobial activity against *E. coli* and *C. perfringens*. Moreover, we aimed to detect certain virulence genes of these pathogens using Multiplex PCR.

MATERIALS AND METHODS

Animals, Feeding and Management: Fifteen clinically healthy male Baladi goat kids, aged 4-6 months and weighing 10-15 kg were used. The kids were not exposed to either stressors or pathogens and were routinely examined clinically according to Radostitis *et al.* [26]. All animals were fed an adaptation period of 2 weeks on constant basal diet which consisted of: 50% yellow corn; 17% wheat bran, 25% cotton seed cake meal, 5% molasses, 2% limestone and 1% salt. Additionally, the animals were supplemented with seasonal green fodders essentially *Alfa Alfa* (Green Barseem) in winter. Bean straw was added at nights while fresh drinking water was offered ad libitum.

Experimental Design: Following a preliminary period of 2 weeks on the basal diet, the goat kids were randomly allocated into three equal groups (n=5):

***Group A*:** Kept as a control and was fed the basal diet only without any feed supplementation.

***Group B*:** Received formulated ration consisted of the basal diet- supplemented with crushed BCS at level of 2%.

***Group C*:** Received formulated ration consisted of the basal diet- supplemented with organic multi-antioxidants: zinc methionine (Zn-Met/10 Zinc chelated with methionine hydroxy analogue – IBEX International) at level of 2 g / kg of diet and Vit. E with Se enriched yeast (E 60.000, Sanovet, Austria – Composition: Vit E 60g, Se yeast 12g, L-lysine 0.08g and carrier: dextrose up to 1 kg)) at the same dose level.

Experimental animal groups were individually housed in separate semi-opened pens and were managed and kept at the same environmental and nutritional conditions throughout the trial which extended for 120 days. Feed ingredients of the experimental diets were well mixed and individually offered at level of 2% of the body weight of the experimental animals, twice at 8.00 a. m. and 2.00 p.m.

Sampling:

Feed Samples: Of experimental diets were collected and chemically analyzed for their nutritive values.

Blood Samples: Venous heparinized blood samples were aseptically collected from all goats at day 0 and once at the end of experimental period for immune cell function tests.

Table 1: Primer sequences for genes F41 and K99 of *E. coli*.

Gene	Primer sequence	Product size	Erf
F41	(F): GCA TCA GCG GCA GTA TCT		
	(R): GTC CCT AGC TCA GTA TTA TCA CCT	380bp	[39]
F5 (K99)	(F): TAT TAT CTT AGG TGG TAT GG		
	(R): GGT ATC CTT TAG CAG CAG TAT TTC	314bp	[39]

Table 2: Primer sequences for the toxin genes (alpha, beta and epsilon) of *C. perfringens*.

Gene	Primer sequence	Product size	Ref
CP ALPHA toxin	(F): GTTGATAGCGCAGGACATGTTAAG		
	(R): CATGTAGTCATCTGTTCCAGCATC	402 bp	[40]
CP BETA toxin	(F): ACTATACAGACAGATCATTCAACC		
	(R):TTAGGAGCAGTTAGAACTACAGAC	236 bp	[40]
CP EPSILON toxin	(F): ACTGCAACTACTACTCATACTGTG		
	(R): CTGGTGCCTTAATAGAAAGACTCC	541 bp	[40]

Fecal Samples: Were aseptically collected from experimental animals at day 0 and subsequently at 30 days intervals up to 120 days of experimental period for bacteriological examination and total bacterial counts as well as detection of certain virulence genes of *E. coli* and toxin genes *of C. perfringens*.

Assays

Chemical Analysis of Experimental Diets: Contents of crude protein (CP) and macro- and micro-elements were determined by standard methods adopted by Association of Official Analytical Chemists [27].

Evaluation of Cellular Immune Responses

Lymphocyte Proliferation Test: Lymphocyte proliferation was measured using XTT assay kit (Cayman Chemical Company, Ann Arbor, MI) as previously described by Sulic *et al*. [28]. The assay is based on the extracellular reduction of the monotetrazolium salts XTT sodium 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)- carbonyl]-2H) into a blue colored- water soluble product (formzan) by the reduced pyridine nucleotide cofactor, NADH, produced in the mitochondria via trans-plasma membrane electron transport and an electron mediator [29]. This conversion takes place in the living cells and is very useful for assaying the cell survival and proliferation. Separation of lymphocytes was applied according to Lee [30] from fresh heparinized whole blood using Ficol-Hypaque (Flow Lab., UK) and the absorbance of the samples was measured using a micro-plate reader (ELISA reader) at a wavelength of 450-500 nm.

Evaluation of Phagocytic Activities of Macrophages Using Candida Albicans: Goat's peripheral blood mononuclear cells were separated by Ficol- Hypaque equilibrium centrifugation method from fresh heparinized blood as described by Mckelvie *et al*. [31].

The phagocytic percentage of Candida ingested was estimated according to the formula described by Harmon and Glisson [32], which was modified by El-Enbaway [33].

$$Phagocytic\ percentage = \frac{No\ of\ phagocytes\ which\ ingest\ Candida}{Total\ no\ of\ phagocytes} \times 100$$

The phagocytic index was calculated according to Richardson and Smith [34].

$$Phagocytic\ index = \frac{Total\ no\ of\ phagocytes\ which\ ingest\ more\ than\ two\ Candida}{Total\ no\ of\ phagocytes\ which\ ingest\ Candida}$$

Total Bacterial Count- Drop Count Method: This method was modified from Collins *et al.* [35] and Proietti *et al.* [36]. Eosine Methylene blue (EMB) and reinforced clostridial agar were used for isolation and enumeration of *E. coli* and *C. perfringens*. EMB plates were incubated at 37°C, aerobically, for 24-48h. The number of grown colonies was determined and the colonies obtained were examined using Gram stain and sub cultured for identification with biochemical tests according to Dragan-Bularda [37]. Reinforced clostridial plates were incubated at 37°C, anaerobically for 24-48h in anaerobic jars (Oxoid) and anaerobic conditions were obtained with Anaerogen (Oxoid). All bacteria were identified using biochemical tests according to Collins *et al*. [35] and Quinn *et al*. [38].

Multiplex PCR: Mixed colonies from each group were tested using multiplex polymerase chain reaction for detection of certain virulence genes of *E. coli* (F41 and F5"K99") and *C. perfringens* ("α", "β" and "ε").

Extraction kit QIA amp mini kit (Qiagen) was used for extraction of bacterial DNA.

Multiplex PCR for *E. coli*: The PCR assay was carried out in a total volume of 50 µl of mixture containing PCR 25 µl of master mix (TAKARA), 1µl from each of the virulence gene-specific primers was taken as shown in Table (1) 5 µl of template DNA and 16 µl dd H2O. The amplification conditions included 25 cycles of a denaturation step at 94°C for 30 s, primer annealing at50°C for 45 s and extension at 70°C for 90 s. The extension time was ramped for an additional 3 s per cycle and a final extension step of 10 min at 70°C was performed. The PCR products were analyzed by electrophoresis through 1.5% agarose gel, after which the gel was stained with ethidium bromide and photographed [39].

Multiplex PCR for *C. perfringens*: The PCR assay was carried out in the PCR reaction mixture (25 µl) contained 5 µl of template DNA, 2.5 µl of 2 mM dNTPs, 2.5 µl 10x PCR buffer, 0.25 µl of 5 µ/µl Taq DNA polymerase (Vivantis, Malaysia), 1 µl from each one of the primers as shown in Table (2) and 6.75 µl dd H2O. Initial denaturation for 5 min. at 94°C, the samples were subjected to 30 cycles of denaturation at 94°C for 1 min., annealing at 55°C for 1 min. and extension at 72°C for 1 min. After the last cycle, a final extension for 10 min. at 72°C was performed. The PCR products were analyzed by electrophoresis through 1.5% agarose gel, after which the gel was stained with ethidium bromide and photographed [40].

Statistical Analysis: Data were analyzed statistically using the ANOVA one way analysis of variance [41].

RESULTS AND DISCUSSION

In this study, we have determined the effect of feeding different forms of antioxidants on cellular immunity in goats. The influence on antibacterial activity against *E. coli* and *C. perfringens* has also been assessed with detection to certain virulence genes of both pathogens.

The experimental diets were formulated by the addition of substantial amounts of different forms of antioxidants to the basal diet. As presented in Table (3), this approach caused differences in the nutritive values of the experimental diets. When compared with the control diet, the experimental diets; 2 and 3 with BCS and multi-antioxidants (Zn-Met. and Vit E with Se enriched yeast) respectively contained more protein and more macro- and micro minerals. These increases were due to the considerable protein and mineral contents of *N. sativa* [25]. Also, dry yeast makes a valuable source of micro elements and amino acids [42]. Diet 3 supplemented with the multi-antioxidants had the highest concentrations of Zn and Se. Based on the 0.1 and 45 ppm recommended for goats, respectively the Se and Zn levels in the basal diet were below the recommended values [43, 44], indicating that supplementation would be warranted. All animals consumed the experimental rations remained clinically normal during the course of the experiment.

Table 3: Chemical analysis and nutritive values of experimental diets on DM basis

	Diet 1*	Diet 2 **	Diet 3 ***
Crude protein (CP) %	13.9	14.50	14.35
Macro- elements %			
Calcium (Ca)	0.70	1.15	0.81
Phosphorous (P)	0.39	0.46	0.42
Magnesium (Mg)	0.27	0.31	0.29
Sodium (Na)	0.54	0.66	0.74
Potassium (K)	0.92	1.20	0.83
Trace elements (ppm)			
Iron (Fe)	135	231	146
Copper (Cu)	8.00	10.00	8.40
Manganese (Mn)	27.00	31.0	29.0
Selenium (Se)	0.058	0.19	0.88
Zinc (Zn)	29.00	43.00	100

*Basal diet (control)

** Formulated diet (Basal diet supplemented with 2% crushed BCS)

*** Formulated diet (Basal diet supplemented with multi-antioxidants; Zn-Met and Vit E with Se enriched yeast at levels of 2g \ kg diet).

Cellular Immune Response of Goats Supplemented with Antioxidants: Oxidant- mediated tissue injury is a particular hazard to the immune system, since phagocytic cells produce reactive oxidants, as a part of the body' s defense against infectious agents. Therefore, adequate amounts of neutralizing antioxidants are required to prevent damage in the immune cells themselves [2]. The results of the present study as shown in Table (4), indicated that BCS and multi-antioxidants significantly ($P<0.05$) enhanced lymphocyte proliferative responses. Similarly, our recorded data demonstrated significant (P<o.o5??) elevation of phagocytic % and phagocytic index in both groups received antioxidants supplement compared with non- supplemented group. Many effects have been reported for the seeds of *N. sativa* and Zn- Met. with Vit. E and Se including their antioxidant roles and immune potentiating effects [2, 3]. This explained the improvement of cellular immune response in the two groups of animals received antioxidants in the current study.

Table 4: Cellular immune response in goat kids dietary supplemented with black cumin seed and multi- antioxidants (Zn- Met. and Vit E with Se enriched yeast) for 120 days compared with control

	Control		Black cumin seed supplement		Multi-antioxidant supplement	
Parameters	Day 0	Day 120	Day 0	Day 120	Day 0	Day 120
Lymphocyte stimulation index	$0.81^{a}\pm 0.01$	$0.83^{a}\pm 0.04$	$0.79^{a}\pm 0.07$	$1.19^{b}\pm 0.10$	$0.73^{a}\pm 0.06$	$0.99^{b}\pm 0.04$
Phagocytic %	$70.73^{a}\pm 2.83$	$69.60^{a}\pm 4.13$	$73.40^{a}\pm 3.51$	$83.08^{b}\pm 3.81$	$68.20^{a}\pm 3.85$	$82.80^{b}\pm 2.07$
Phagocytic index	$0.62^{a}\pm 0.04$	$0.59^{a}\pm 0.04$	$0.56^{a}\pm 0.07$	$0.79^{b}\pm 0.04$	$0.60^{a}\pm 0.02$	$0.80^{b}\pm 0.03$

Data are presented as mean ± SE

Mean values with different superscript letters are significantly different at $P<0.05$.

Data regarding the effect of dietary BCS on immune response are in agreement with those recorded by El Bagir *et al.* [23] in rabbits. They found that blood from donor rabbits fed diets containing BCS had increased phagocytic activity against *Staphylococcus aureus*. Similar observations have been made in human patients with allergic rhinitis. The treatment with allergen specific immunotherapy plus intake of BCS produced a significantly greater increase in phagocytic activity of polymorphonuclear leukocytes than immunotherapy alone [45].

Although the therapeutic mechanism of *N. sativa* is unknown, the presence of the trace elements that are essential nutrients and important for immune functioning, such as Fe, Cu, Zn and Mn, suggests one possible mechanism [25]. It is likely that BCS contains a factor that stimulates the polymorphonuclear leukocyte phagocytic activity and the lymphocyte response to various mitogens [46].

With regard to multi-antioxidants, our results are in accordance with those presented earlier by Finch and Turner [47] in which Vit. E and Se supplementation resulted in enhanced lymphocyte responses in lambs. Also, Ndiweni and Finch [48] found that Vit. E and Se enhance the function of macrophage with *Staph aureus*. On the same line, dietary supplementation of horses with Vit E and Se [49] resulted in improvement of lymphocyte proliferative response and significant elevation of phagocytic and killing activities.

Recently, Hamam and Abou- Zeina [7] reported that ewes supplemented with vitamin. E and Se has better antioxidants status and greater immunoglobulins. It was concluded that vitamin E and Se supplementation improve the status of these micronutrients and humoral immune response in buffalo calves [15].

Meanwhile, Abou- Zeina *et al.* [16] demonstrated that Zn, Se and Vit E administered to buffalo cows under heat stress condition, induced better reproductive performance and increase in immunoglobulin levels.

How Se and Vit E enhance the immune response is not fully understood. It is possible that Se as a cofactor of the GPx family of enzymes, either through a direct effect or via destruction of inhibitory peroxides, contributes to a better immune response [10]. As a constituent of selenoproteins, Se is needed for the proper functioning of neutrophils, macrophages, natural killer cells and T lymphocytes [5]. Mild Se deficiency appears to increase susceptibility to oxidant stress [50] and risk of infections. It was documented by many authors that deficiency of Se also affects the ability of the macrophages to release the leukotriene B4 which signals the white blood cells to aid in the destruction of harmful microorganisms [51]. The antioxidant activity of vitamin E in preventing lipid oxidation may be one of the mechanisms by which vitamin E enhances immunity particularly phagocytosis [11]. Besides, Vitamin E causes a reduction of glucocorticoids, which are known to be immunosuppressive. Researchers suggested that relatively higher levels of vitamin E supplementation may improve animal performance, which may be due to enhanced immunity [15, 52].

Likewise, Zinc is crucial for normal development and function of cells mediating nonspecific immunity such as neutrophils and natural killer cells [53]. Zinc also, regulates the expression in lymphocytes of metallothionein and metallothionein-like proteins with antioxidant activity. *In vitro* intracellular killing by macrophages is very sensitive to zinc deficiency and is restored rapidly after supplementation. Zinc deficiency adversely affects the secretion and functions of cytokines, the basic messengers of the immune system [13].

Antimicrobial Activity of Antioxidants Against *E. coli* and *C. perfringens*: The effect of supplying goat kids with BCS and multi-antioxidants on total bacterial counts (TBC) of *E. coli* and *C. perfringens* in fecal samples were evaluated in sixty fecal samples collected and cultured five times through 120 days. A remarkable decline in both

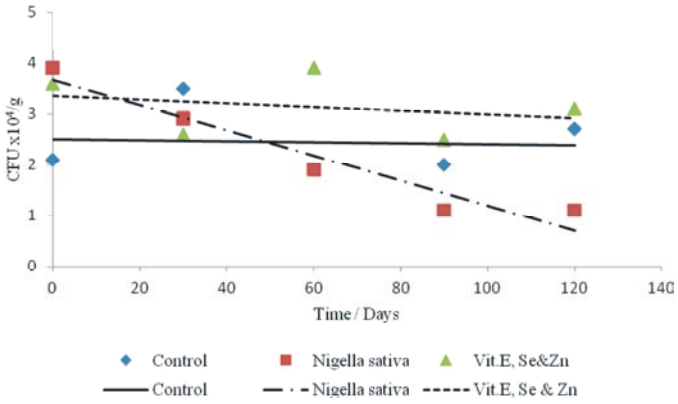

Fig. 1: *E. coli* count from samples of goats kids

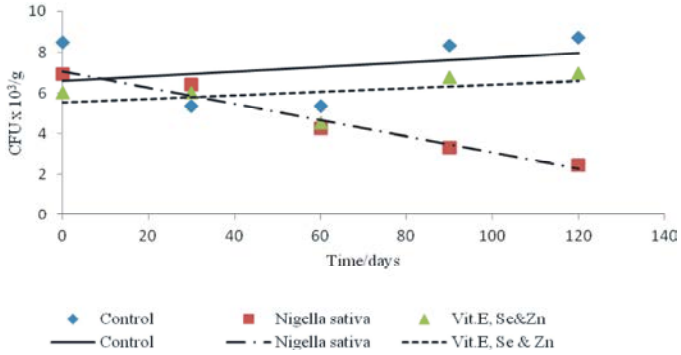

Fig. 2: Clostridia count in fecal samples from goat kids

counts of *E. coli* and *C. perfringens* after feeding goats on ration supplemented with BCS (*N. sativa*) was detected (Fig. 1 & 2). This result is in harmony with those obtained by [19, 22, 54, 55] who reported that the oil fraction of BCS contains thymoquinone which exerts antibacterial, antioxidant and immunopotentiating activities via increasing neutrophil percentage and hence increasing the defense mechanism of the body against infection. That grew more interest in *N. sativa* and soon after that was reported to possess anti-bacterial activity against *Listeria monocytogenes* [56] and promote wound healing in farm animals [57]. It was observed that the feeding of BCS elongated survival time of rabbits after the intraperitoneal administration of *Pasteurella multocida* [23]. This observation corroborates an earlier study in mice infected with *Staphylococcus aureus*, showing eradication of the infection by the simultaneous injection of a diethyl-ether extract of *N. sativa* seeds [58].

Zinc and Vit E with Se could improve the immunity of animals and protect them from appearance of clinical signs of disease although, pathogenic bacteria was detected. Combination of vitamin E and selenium improve cell mediated and humoral immune response and increased resistance to *E. coli* infection [59].

Multiplex PCR. Detection of certain virulent genes of *E. coli* (K99 and F41) using multiplex PCR, applied on pool mixed colonies from each group, showed positive results to "K99" at 314 bp amplicon in all samples (Photo 1). Fimbrial adhesion "K99" plays a role in colonization of small intestine epithelial cell of enterotoxigenic *E. coli* [55]. The oil fraction of BCS contains thymoquinone which exerts antibacterial activity and may have direct effect against colonization of *E. coli* K99 in the small intestine explaining the marked decrease in the total bacterial counts [60]. Enterotoxigenic *E. coli* (ETEC) strains are very important pathogen because they adhere to the small intestinal microvilli without inducing morphological lesions and produce enterotoxins acting locally on enterocytes. This action results in the hyper secretion of water and electrolytes and reduced absorption [39]. Adhesions and toxins are the two prominent virulence attributes of ETEC and the level of knowledge of these factors determines the chances for successful prevention and therapy of the disease. For animal ETEC the most common adhesions are the fimbriae: F41 and F5 (K99) [61]. The study used PCR as specific, sensitive and epidemiologic tools for the detection of ETEC.

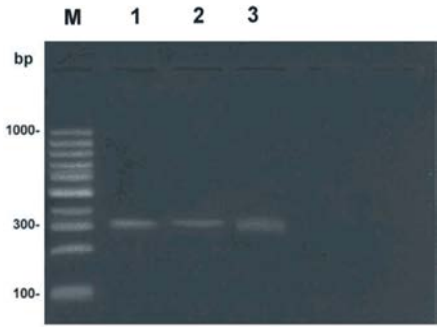

Photo 1: Amplified PCR product of (*K99*) gene (314 bp) by multiplex PCR. Lane M: 100bp ladder, Lane 1, 2and 3 are *K99* positive *E. coli* strains

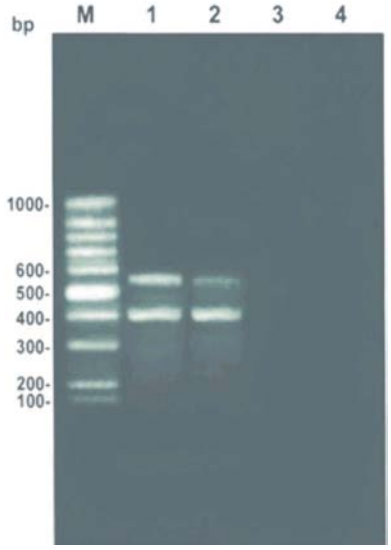

Photo 2: Amplified PCR product of alpha (402 bp) and epsilon (541 bp) genes by multiplex PCR. Lane M: 100 bp ladder, Lanes 1and 2 are positive for *C. perfringens* type D.

Detection of certain virulent toxin genes *C. perfringens* ("α", "β" and "ε") using multiplex PCR was applied on pool mixed colonies from each group. The result of multiplex PCR showed positive results of alpha at (402 bp) and epsilon at (541 bp) genes of *C. perfringens type D* (Photo 2). *Cl. perfringens* type D is the etiological agent of enterotoxaemia (pulpy kidney disease) of several animal species [26]. Epsilon toxin, a major exotoxin produced by this microorganism [62], can be absorbed through the intestinal mucosa [63] and then spread via the circulation to internal organs, where they cause fluid accumulation in body cavities and edema in several organs, notably brain, heart, lungs, liver and kidney [64]. Type D enterotoxemias can result in peracute, acute or chronic disease [63].

On the basis of the present results, it can be concluded that dietary antioxidants from natural herbs such as *N. sativa* and organic multi nutrient-antioxidants (Zn- Met. and Vit E with Se enriched yeast) have potential for improvement of cellular immune responses of goats. Moreover, *N. sativa* showed antimicrobial effect against some enteric pathogens by inhibition of the intestinal bacterial counts of *E. coli* and *C. Perfringens.* Using Multiplex PCR, positive results of K99 gene was detected for *E. coli* while, alpha and epsilon toxin genes were detected for *C. perfringens* type D.

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