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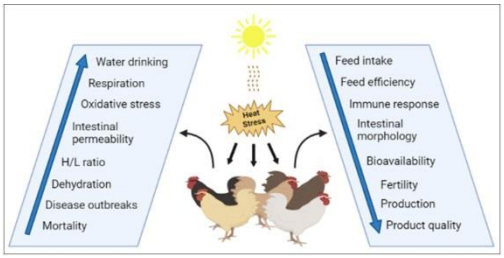
Review

Nutritional advances in production performance and product quality of poultry husbandry under heat stress

Ali Shah SR, and Çetingül IS.

Online J. Anim. Feed Res., 12(2): 53-65, 2022; pii: S222877012200008-12
DOI: https://dx.doi.org/10.51227/ojafr.2022.8

Abstract



Ali Shah SR, and Çetingül IS (2022). **Nutritional advances in production performance and product quality of poultry husbandry under heat stress.** *Online J. Anim. Feed Res.*, 12(2): 53-65. DOI: https://dx.doi.org/10.51227/ojafr.2022.8

The objective of this review is to explore the nutritional additives to combat heat stress in high production targeted fowls. Many diseases and stress dynamics are liable for heavy mortality nowadays. High production targets and heat stress initiate free radical injuries and gastrointestinal oxidative insults resulting in poor bioavailability and feed efficiency. Accordingly, the quality of the eggs and meat is compromised along with the bird's health. Dietary schemes and relevant feed formulation with the provision of vital elements like selenium, zinc, calcium, vitamin E, vitamin C, vitamin A, electrolytes, essential amino acids, and plant extracts can conflict with all sorts of stress in birds and improve the immune system. The occurrence of many contagious diseases, nervous ailments, and metabolic syndromes can be decreased via appropriate feeding routines. It will not only control the bird's health but also increase the quality and market value of the products and consumer satisfaction. Scientists have struggled to prevent immuno-depression, egg and meat quality impairments by dietary influences. Poultry welfare is facing many problems currently which should be properly coped with innovative nutritional maneuvers. This review anticipates illuminating the probable nutritional approaches to manage stress in poultry birds.

Keywords: Heat stress, Poultry, Electrolytes, Phytogenic, Product quality

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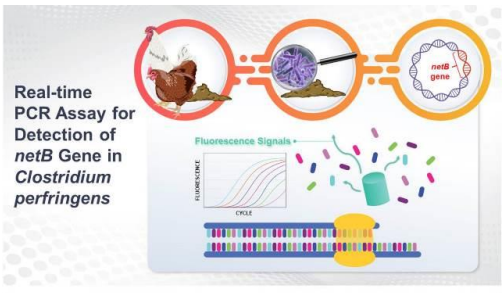
Research Paper

Development of a real-time PCR assay for the rapid detection of *netB* gene in *Clostridium perfringens*

Soo GH, Tan J, and Ong HCh.

Online J. Anim. Feed Res., 12(2): 66-72, 2022; pii: S222877012200009-12
DOI: https://dx.doi.org/10.51227/ojafr.2022.9

Abstract



Soo GH, Tan J, and Ong HCh (2022). **Development of a real-time PCR assay for the rapid detection of *netB* gene in *Clostridium perfringens*.** *Online J. Anim. Feed Res.*, 12(2): 66-72. DOI: https://dx.doi.org/10.51227/ojafr.2022.9

Necrotic enteritis (NE) has a huge economic impact in the poultry industry. Numerous studies have shown that necrotic enteritis toxin B (*net*B) is a key virulence factor in *Clostridium perfringens* strains that cause NE in chickens. Early detection of *netB* in *C. perfringens* is important to circumvent the spread of NE. In this study, we developed a novel combinatorial approach involving (i) a commercial 2-step DNA extraction kit and (ii) a real-time polymerase chain reaction (qPCR) for the detection of *netB*-positive *C. perfringens* in fecal samples. Melt curve analysis and specificity test demonstrated 100% specificity without any cross-reactivity in other bacterial species with a limit of detection of 10^2 cfu/g. Field validation was subsequently conducted on nine fecal composites collected from different layer houses at two commercial farms, leading to successful detection of four *netB* positive samples. The study presented a rapid diagnostic qPCR assay involving a 2-step DNA extraction protocol to screen for *C. perfringens* carrying *netB* gene in chicken fecal samples.

Keywords: *Clostridium perfringens;* Necrotic enteritis; *netB* gene; Poultry; Real-time PCR

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Research Paper

The impact of stable flies (*Stomoxys calcitrans* L.) on small stock production in Bodibeng, Bothatogo and Sehithwa in the North West district, Botswana; a survey study

Moreki JC, Tjinyeka K, Makore J, Tlotleng K, Moseki MI.

Online J. Anim. Feed Res., 12(2): 73-80, 2022; pii: S222877012200010-12
DOI: https://dx.doi.org/10.51227/ojafr.2022.10

Moreki JC, Tjinyeka K, Makore J, Tlotleng K, Moseki MI (2022). **The impact of stable flies (*Stomoxys calcitrans* L.) on small stock production in Bodibeng, Bothatogo and Sehithwa in the North West district, Botswana; a survey study.** *Online J. Anim. Feed Res.*, 12(2): 73-80. DOI: https://dx.doi.org/10.51227/ojafr.2022.10

Abstract
Stable fly, *Stomoxys calcitrans* L. (Diptera: Muscidae) is a globally recognized livestock pest of economic importance, which also attacks wild animals, pets and humans. These flies frequently feed on the forelegs of animals and can cause significant production losses and severe animal health and welfare concerns. This study investigated the impact of stable flies on small stock (sheep and goats) production and documented control measures adopted by farmers in Sehithwa, Bodibeng and Bothatogo villages in the North West District of Botswana. Simple Random Sampling was used to select 90 respondents in the study area. Data on demographic characteristics (i.e., age, sex, marital and educational status of the respondents), control measures against stable flies, time stable flies appeared, factors contributing to abundance of stable flies, and the role of government and private sector in the control of stable flies were collected and analysed using SAS. The Chi-square test of goodness of fit was used to show the unequal distribution of the frequencies of respondents among the categories for each variable. Results showed that wood smoking (53.33%) was the common control measure against stable flies followed by migration to unaffected areas (35.55%) and dipping (11.11%). Feeding activity of stable flies reached its peak in the evening (58.89%) followed by morning (31.11%), afternoon (6.67%) and the least was night (3.33%). Eighty-seven percent of respondents mentioned that stable fly contributed to poverty, starvation (16.67%) and loss of income (13.33%). It is concluded that stable flies affect livestock productivity and people' livelihood; hence the need to adopt effective control measures. Control measures against these flies will be more effective when applied in the evening and morning.

Keywords: Control measures, Economic losses, Livestock pest, Small stock, Stable flies.

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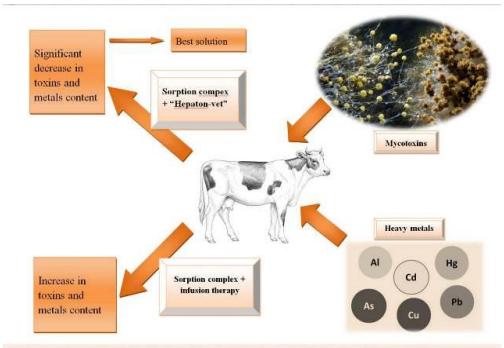
Research Paper

New methods for detoxification of heavy metals and mycotoxins in dairy cows

Baryshev VA, Popova OS, and Ponamarev VS.

Online J. Anim. Feed Res., 12(2): 81-88, 2022; pii: S222877012200011-12
DOI: https://dx.doi.org/10.51227/ojafr.2022.11



Baryshev VA, Popova OS, and Ponamarev VS (2022). **New methods for detoxification of heavy metals and mycotoxins in dairy cows.** *Online J. Anim. Feed Res.*, 12(2): 81-88. DOI: https://dx.doi.org/10.51227/ojafr.2022.11

Abstract
Among the many environmental and industrial factors that adversely affect the soil, the pollution with heavy metals and mycotoxins occupies a special place in livestock breeding. This study aimed to demonstrate methods of treating and pharmacological correction of a toxic state intensified by heavy metals and mycotoxins in cattle using native drug as mycotoxin-deactivating feed additive. A total of 20 highly productive Holstein cows aged 3-4 years were divided into 2 groups, based on clinical and biochemical parameters. The experimental group (n=10) fed a diet supplemented with 4% sorption complex and a drug based on *Silybum marianum* and ursodeoxycholic acid as a hepatoprotector named "Hepaton-vet". The control group (n=10) was injected with a complex of sorbents (consisting of perlite, vermiculite, and polyphepan in equal proportions) at a dose of 4% of the daily intake of food, and the rest of the treatment was carried out with the help of daily infusion therapy. So in the feed samples, only the T-2 and Deoxynivalenol (DON) indicators exceeded the normal value by 1.66% and, 3%, respectively. Thus, the practical efficiency concerning T-2, aflatoxin and Deoxynivalenol were to 100%, 86%, 18%, respectively. Cadmium in compound feed was lower by 44%, in comparison with the maximum permissible concentration, followed by 53.3% in hay and 78% in silage. The amount of lead in compound feed and silage was 78%, and it was 35% in the hay. In conclusion, the use of a complex of sorbents, together with newly developed component "Hepaton-vet", led to positive results, allowing for the identification of several effects that influenced the metabolic processes in the liver, which was confirmed by the results of morpho-biochemical blood tests and clinical diagnostics of the animals' condition.

Keywords: Cattle, Heavy metals, Mycotoxins, Silage, Sorption complex.

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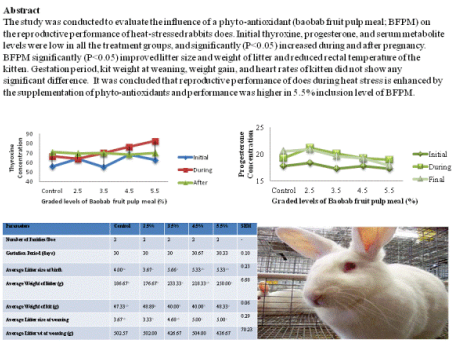
Research Paper

Effect of supplementation of phyto-antioxidant (baobab fruit pulp meal) on reproductive performance of rabbit does during heat stress

Anoh KU, Paul JN and Amu UM.

Online J. Anim. Feed Res., 12(2): 89-96, 2022; pii: S222877012200012-12
DOI: https://dx.doi.org/10.51227/ojafr.2022.12

Abstract
A study was conducted to evaluate the influence of a phyto-antioxidant (baobab fruit pulp meal; BFPM) on the reproductive performance of heat-stressed rabbits does. A total of 50 New Zealand White crosses adult rabbit does of 12 months old were used in the present study. The rabbits were randomly allotted into five experimental treatment groups, with ten rabbits per treatment in a completely randomized design. The rabbits were fed diets supplemented with graded levels (0.0%, 2.5%, 3.5%, 4.5% and 5.5%) of BFPM. Parameters monitored were thyroxin and progesterone secretion, serum metabolites, reproductive traits, and physiological performance of the kitten. Initial thyroxin, progesterone, and serum metabolite levels were low in all the treatment groups, and significantly increased during and after pregnancy. BFPM significantly improved litter size and weight of litter and reduced rectal temperature of the kitten. Gestation period, kit weight at weaning, weight gain, and heart rates of kitten did not show any significant difference. It was concluded that reproductive performance of does during heat stress is enhanced by the supplementation of phyto-antioxidants and performance was higher in 5.5% inclusion level of BFPM.
Keywords: Antioxidant, Gestation, Heat stress, Rabbit, Reproductive hormones.



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Research Paper

Nutritional value and in situ degradability of selected forages, browse trees and agro industrial by-products

Terefe G, Faji M and Mengistu G.

Online J. Anim. Feed Res., 12(2): 97-102, 2022; pii: S222877012200013-12
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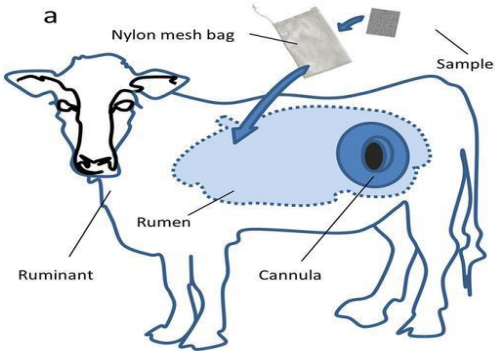
Abstract
The *in situ* dry matter and crude protein degradability of grasses, legumes, browse trees and agro industrial by products was evaluated by the fistulated bulls (Boran × Holstein-Friesian with mean body weight 580 kg and age= 29±3 months). The lower (P < 0.05) crude protein content was reported in bracharia grasses than the other grasses. The higher (P < 0.05) washing loss in bracharia and Rhodes grasses and the better (P < 0.05) potential and effective degradability for dry matter (DM) and crude protein (CP) was observed in desho grass compared with other grasses. The content of crude protein, relative feed value and potential DM degradability were higher (P < 0.05) in Sesbania than Pigeon pea and tree lucerne. Potential and effective DM degradability was better (P < 0.05) in tree lucerne than the other browses. *Acacia nilotica* and Wanza (*Cordia africana*) had the greater (P < 0.05) washes loss, potential and effective degradability for dry matter and crude protein than the other browse trees. Cactus and Shola had the highest (P < 0.05) undegradable protein than *Acacia nilotica* and Wanza. The two energy source feeds (maize and wheat bran) had the greater (P < 0.05) potential and effective dry matter degradability than the other by products. The rumen undegradable protein was higher in vetch than lablab. The in situ dry matter degradability values obtained in this study can be useful to identifying the best materials used ruminant feeds.
Keywords: By-products, Dry matter, Forage, Rumen degradability, Bulls.



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NUTRITIONAL ADVANCES IN PRODUCTION PERFORMANCE AND PRODUCT QUALITY OF POULTRY HUSBANDRY UNDER HEAT STRESS

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Supporting Information

REVIEW ARTICLE

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ABSTRACT: The objective of this review is to explore the nutritional additives to combat heat stress in high production targeted fowls. Many diseases and stress dynamics are liable for heavy mortality nowadays. High production targets and heat stress initiate free radical injuries and gastrointestinal oxidative insults resulting in poor bioavailability and feed efficiency. Accordingly, the quality of the eggs and meat is compromised along with the bird's health. Dietary schemes and relevant feed formulation with the provision of vital elements like selenium, zinc, calcium, vitamin E, vitamin C, vitamin A, electrolytes, essential amino acids, and plant extracts can conflict with all sorts of stress in birds and improve the immune system. The occurrence of many contagious diseases, nervous ailments, and metabolic syndromes can be decreased via appropriate feeding routines. It will not only control the bird's health but also increase the quality and market value of the products and consumer satisfaction. Scientists have struggled to prevent immuno-depression, egg and meat quality impairments by dietary influences. Poultry welfare is facing many problems currently which should be properly coped with innovative nutritional maneuvers. This review anticipates illuminating the probable nutritional approaches to manage stress in poultry birds.

Keywords: Heat stress, Poultry, Electrolytes, Phytogenic, Product quality

INTRODUCTION

The poultry sector faces several stress dynamics including feather pecking, vaccination, molting, increased stocking density, and high temperature. Although sometimes stress seems to be harmless, continuing stress circumstances can undesirably upset the vigor and production of the birds. Poultry birds need more nutrient requirements and metabolic rate is higher as compared to ruminants (Akşit et al., 2006). Poultry birds are very prone to heat stress as compared to other farm animals. Commercial broilers produce more metabolic heat as compared to other indigenous pure genotypes (Kang and Shim, 2021). Under heat stress, feed intake, feed conversion ratio, productivity, meat quality, egg quality, and immune status are compromised. Heat stress is a primary menace that deleteriously changes bird performance (Liu et al., 2020). Poultry birds uphold their body temperature at 40.6–41.0°C regardless of the ecological high temperature because of the homoeothermic activity (Ryder et al., 2004). Nevertheless, birds, whose bodies are covered with plumages and raised for high productivity are extra profound to heat stress (Mahmoud et al., 2015). Broiler meat and eggs are the most cost-effective animal protein nowadays. Globally poultry farming will progressively be exaggerated as birds are unprotected to periodic and prolonged heat stress associated with global warming (Wankhade et al., 2020).

The optimal temperature for the best productivity of birds is 19-22°C for laying hens and 18-22°C for growing broilers (Shahzad et al., 2021). High temperature in poultry causes reduced fertility, growth and production, poor quality meat and egg production, and more mortality (Ranjan et al., 2019). Furthermore, genetically modified layers kept for high production generate more metabolic heat so they are more prone to heat stress (Holik, 2015).

For the period of initial summertime, the poultry husbandry faces considerable damages in production, and particularly pale, soft, exudative (PSE) meat. PSE meat has a low water-holding ability and meat consistency. Certainly, these fluctuations are strictly interrelated to biochemical variations of skeletal muscles due to heat stress (Song and King, 2015). Immunity (Siddiqui et al., 2021), production, and bird welfare are undesirably affected due to heat stress in birds (Iyasere et al., 2021). The major reason for production loss due to stress is less feed intake of the birds and for every 1°C after 30°C, almost 5% feed intake drops which results in essential nutrient deficiencies (Bilal et al., 2021). To cope up with this, the supplementation of vitamin C and E, chromium, zinc, and methionine, high protein and energy, phytogenic extracts can be operative in decreasing the deleterious effects of heat stress (Khan et al., 2014; Surai et al., 2019).

This review is considered to anticipate the importance of nutrition in responding to the negative effects of heat stress on poultry production. Maximum of the possibilities and remedies of heat stress and negative impacts are illustrated.

Citation: Ali Shah SR, and Çetingül IS (2022). Nutritional advances in production performance and product quality of poultry husbandry under heat stress. *Online J. Anim. Feed Res.*, 12(2): 53-65. DOI: https://dx.doi.org/10.51227/ojafr.2022.8

DISCUSSION

Feeding and drinking response during stress

Undoubtedly feed intake decreases significantly when housing temperature upsurges. In a study, it was determined that feed intake was reduced and water intake was increased in broiler chicks and this behavior was in response to high metabolic heat (Chowdhury and Furuse, 2013). As a thumb rule, birds can be nourished at midnight during hot weather. Nevertheless, to avoid high metabolic heat overlapping ecological heat, it is usually recommended not to feed at 1.00-4.00 pm. Feed conversion rate or feed efficiency of the birds is reported to increase during heat stress periods irrespective of the feed intake and crop filling (Sohail et al., 2012). In a study, it was concluded that if broilers eat more finely ground feed particles during stress time, little metabolic heat is produced as compared to coarse particles of maize and the birds adapt to the environment with panting (Santos et al., 2019).

A study was conducted on 3060 broilers kept in tropical, subtropical, and temperate areas. FCR and body weight at 42 days were considerably higher ($p \leq 0.05$) in the subtropical area during winter (Osti et al., 2017). Temporary feed restriction appears to improve the productivity and feed efficiency of the birds during the hot season. A study was conducted on one-week-old broiler chicks kept in open housing and it was seen that feed restriction for 3 hours along with vitamins and mineral supplementation reduced mortality and improved production and quality of the meat (Mohamed et al., 2019). Similarly in another study, feed restriction for 2 hours per day improved the growth performance in broiler chicks (Liew et al., 2003). Conversely, feed restriction for a longer period decreased considerably the body weight gain and feed efficiency of broilers but reduced mortality (Lin et al., 2006).

It was shown in a study conducted on various genetic lines of broilers kept in semi-intensive housing and the results showed that all chicks showed maximum feeding intake at early morning 7:00 and late evening 17:00 when the temperature is decreased (Gonçalves et al., 2017). Hens under heat stress walk very little drink more water and do more rest as compared to control (Mack et al., 2013). The increase in water intake under stress is a physiological response of birds to homeostat body temperature and dehydration. Similarly in a study betaine was used in broilers due to its osmoregulation property to decrease heat damage and increase water intake (Ratriyanto and Mosenthin, 2018). The most economical way to decrease stress effects on birds is to feed them in the early morning and late evening and this will enhance feed efficiency (Liverpool-Tasie et al., 2019). Birds eat more in day time normally and less at night time but to compensate for the stress-induced decrease in feed intake in the daytime, nighttime feeding can be encouraged with synthetic illumination (Daghir, 2009).

Negative impacts of heat stress on poultry

Due to oxidative stress and cellular injuries during heat stress immunity and feed efficiency of the birds drops. There happens massive economic loss due to less production and mortality. The negative effects of heat stress on poultry are shown in figure 1 and table 1.

Nutritional intermediations improving product quality and production

Nutritional management is an auspicious way to enhance productivity, feed efficiency and reduce disease outbreaks by increasing the immunity of the birds during stress (Mujahid, 2011). Nutritional strategies include balancing of energy, provision of essential amino acids, adequate minerals and vitamins and many phytogenics that are discussed below and shown in figure 2.

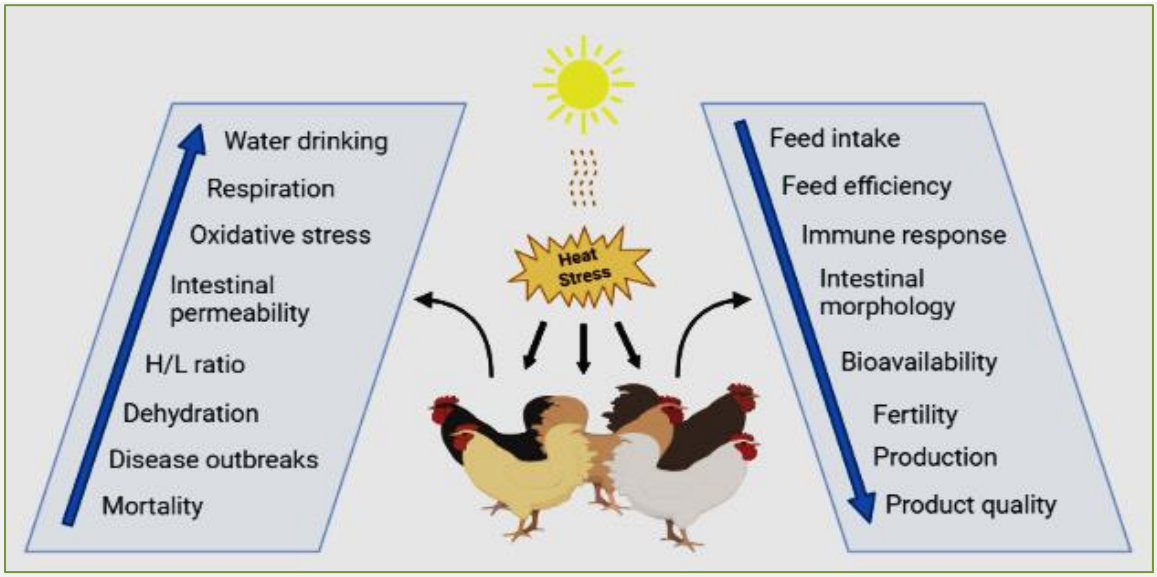

Figure 1 - Negative impacts of heat stress

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Table 1 – The negative impacts of heat stress

Heat Stress	Effects	Birds	References
Chronic heat stress (10-days)	↓ Weight gain ↑ Inflammatory cytokines (IL-2 and TNF-α), and rectal Temperatures. ↑ Intestinal bacterial permeability	Broilers	Alhenaky et al. (2017)
Chronic heat stress	↓ Feed intake and body weight.	Cobb-500 broiler	Attia et al. (2017)
Acute heat stress	↑ Mitochondrial Super-Oxide formation in bird's skeletal muscles.	Broilers	Sahin and smith (2016)
38°C (2, 5, and 10 hr.)	An efficient ↑ TLR4 expression in kidneys, liver, and heart.	28 days old broilers	Huang (2017)
23.9-37°C Cycling	↑ Heterophil-lymphocyte ratio, serum levels of cholesterol, glucose, and triglycerides. ↓ Feed intake, body and organ weight, primary and secondary antibody and IgM and IgG titers, HDL-cholesterol (serum concentration).	Broilers	Habibian et al. (2014)
31°C (12 HR.)	↓ In laying rate and egg production. ↑ (In mRNA levels) of ghrelin and amphetamine- and cocaine-regulated	12 weeks old hens	Song et al., (2012)
30°C (2 weeks)	↑ Serum corticosterone conc., and sodium-dependent glucose transporter-1 expression. ↓ Bodyweight.	28 days old broilers	Garriga et al. (2006)
32°C (acute heat stress)	Acid-base level disturbance (in the blood), negative effects on the integrity of muscle membrane, and changes in meat characteristics.	35-63 days old broilers	Sandercock et al. (2001)

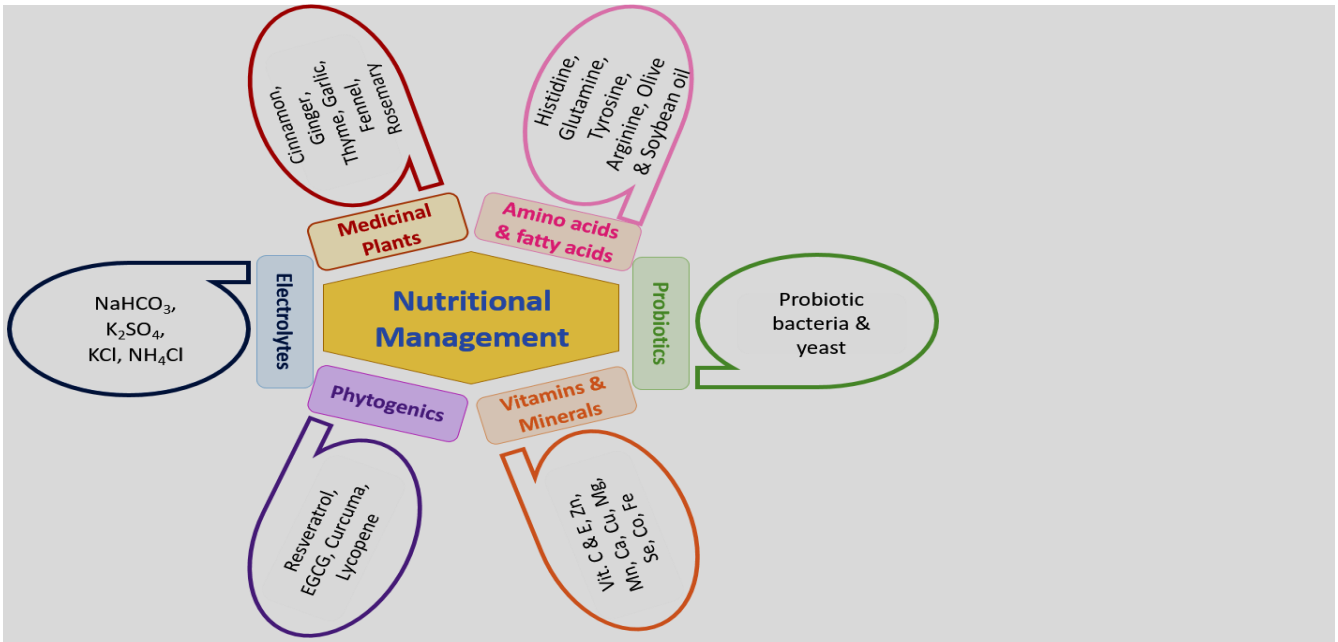

Figure 2 - Nutritional strategies for decreasing heat stress

High-energy formulations

The benefits of giving high-energy formulations by supplementing fat and protein to broilers kept in high-temperature areas are familiar globally (Srinivasa Rao et al., 2016). For instance, a study was conducted on Ross-308 and chicks were fed with 21% and 23% crude proteins. The results showed a significant increase ($P< 0.05$) in body weight gain and feed conversion ratio as compared to the low protein diet. In short, it was declared that 23% protein and 3000 kcal/kg energy was better for growing stage and for the finishing period 20% protein and 3200 kcal/kg energy was suitable (Ahmed and Arabi, 2015). Feeding broilers with less protein-based diets during finishing has reduced microbial contents in feces and improved productivity during stress (Laudadio et al., 2012). In another study, for layers kept at 31 °C, supplementation of 5% fats to layers increased their feed intake by 17% while for hens kept at 11-18°C, there was a 4.5% increase in feed intake (Daghir, 2008). Abdel-Moneim et al. (2020) reported that full-fat canola meal in Japanese quails under heat stress has resulted in improved growth and egg-laying along with egg quality.

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Lowering metabolic heat

As compared to carbohydrates and protein, supplementary fat leads to a slight rise in metabolic heat (Laganá et al., 2007). Serving wet feed to broilers during stressful environmental conditions increases weight gain, feed conversion rate, and feed intake (Koh and Macleod, 1999). Mashed feed or crumbled feed particles are easily digested with little effort of gizzard i.e. high digestibility and hence reduced metabolic heat is produced (Amerah et al., 2007).

Betaine and methyl donors' supplementation

Betaine has good osmoregulation properties and its supplementation to quails at 0.06-0.12% has shown more feed intake and better egg quality during heat stress (Ratriyanto et al., 2017). Similarly in another study on quails, 16.5, 18, and 19.5% protein levels were augmented with 0, 0.06, 0.12% betaine respectively. The results showed that 18% protein with 0.12% betaine showed maximum crude fiber digestibility but less fat digestibility. Betaine improved all egg quality parameters and improved the digestibility of all nutrients (Raei et al., 2020). In another study, betaine was used in broiler chicks to decrease harmful effects of heat stress and it resulted in decreased mortality, improved body weight gain and feed intake, and humoral immune response of the chicks under stress in treatment groups as compared to the control (Ghasemi and Nari, 2020).

Reduced use of usual amino acids in diets during heat stress with supplementation of methionine, lysine, and betaine can improve the feed intake and efficiency along with a reduction in metabolic heat (Cronje, 2018; He et al., 2021). Moreover, these methyl group donors have value-added the carcass traits of broilers during heat stress (Ratriyanto et al., 2014). Glutamine supplementation in broilers under heat stress has resulted in intestinal epithelial cells multiplication increasing intestinal barrier function and tight junction proteins (Wu et al., 2018).

Taurine supplementation

Taurine has now made its position in animal nutrition as an antioxidant agent because of stress in poultry birds. In a study, taurine was used in broilers in drinking water for 35 days during heat stress. The results showed that there was a significant increase in weight gain and feed intake in taurine supplemented groups as compared to control. The gut fat was reduced along with improvement in muscle histopathological characters (Hafeez et al., 2021).

Probiotics and prebiotics supplementation

Probiotics and prebiotics are very valuable in poultry farming under heat stress. In laying hens provided with a high-temperature environment, 1g of mannan-oligosaccharides was used and it resulted in improvement of weight gain and feed conversion ratio, eggshell weight and thickness, albumen weight, and height in treatment groups (Bozkurt et al., 2012). Similarly, in broilers under heat stress, 24mg/kg of the diet was used for 42 days and it resulted in improvement of intestinal morphometric characters including villus width and surface area, and crypt depth and production of the birds (sohail *et al.* 2012). In laying hens probiotic mixture including 0.1% symbiotic and 0.1% culture of *Bacillus subtilis and Enterococcus faecium* and 0.5% mannan-oligosaccharide was used and it resulted in daily feed intake increase along with an increase in egg weight, egg-laying capacity, and egg quality parameters under heat stress. It also enhanced beneficial intestinal microbes (Zhang et al., 2017). In commercial broilers, strains of *Bacillus subtilis* were used and it increased daily body weight gain and lipid peroxidation reduction (Chao et al., 2017). Similarly, dietary supplementation of *Bacillus subtilis* at 0, 200 and 400 mg/kg of feed in broilers under heat stress increased significantly IgM levels in serum and improved cell-mediated immunity (Fathi et al., 2017). In a study, microalgae astaxanthin obtained from *Haematococcus pluvialis* was used in broilers and layers to evaluate its effects on molecular parameters of stress and lipid metabolism during heat stress and it decreased hepatic mRNA activity of many redox regulatory genes, heat shock transcription factor 1, heat shock protein 70 and tumor necrosis factor-α (Tolba et al., 2020). In a study, post-biotic obtained from *Lactiplantibacillus plantarum* was used in broilers to decrease the negative impacts of heat stress on production and bird's health. It resulted in upregulation of the glutathione peroxidase, superoxide dismutase, and catalase activity so it is a natural source of anti-oxidation (Humam et al., 2021).

Electrolytes supplementation

Electrolytes are very important for poultry that should be balanced during heat stress and it is recommended to be 250 mEq/kg during stressful conditions (Sarwar, 2007). For better productivity of broilers during heat stress, 0.21-0.25% sodium and 0.30% chloride should be provided during the finishing stage (Mushtaq et al., 2007). In a meta-analysis, it was predicted that for every 1°C increase in ambient temperature there was a 1.4% reduction in average daily feed intake and 2.1% reduction in average daily weight gain and dietary electrolyte balance has a strong effect on chicks less than 21 days old (Andretta et al., 2021). Due to decreased feed intake during heat stress, there comes dietary deficiency of vital minerals that are important for digestibility and oxidative stress neutralization. Hence, there should be controlled supplementation of calcium, zinc, iron, phosphorous, sodium, chloride, potassium, copper, magnesium, selenium, manganese, chromium, and iodine (Hassan Mir et al., 2018). In a study, broilers during the finishing stage were provided with minerals in water and feed during heat stress maintaining the electrolyte balance as 240 mEq. The results showed a 34% increase in water consumption as compared to control. Moreover, mineral supplementation reduced significantly metabolic heat, hyperventilation, and mortality (Farfán et al., 2010).

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Vitamin C and vitamin E supplementation

Birds open to high-temperature present fall in the size of immune organs like the bursa, thymus, and spleen. Oxidative damage happens in birds prone to heat stress so, dietary addition of antioxidants is common dietary involvement. The provision of 250 mg/kg of vitamin E has been seen to decrease undesirable effects that occur during heat stress (Kazim Sahin et al., 2001). In another study, vitamin E was supplemented as 250 mg/kg of the diet in broilers and it resulted in increased activity of glutathione peroxidase and reduced expression of HSP60 (Kumbhar et al., 2018), and in layers, at 150 mg/kg it has shown to increase egg number and quality (Zhao et al., 2011). Ascorbic acid or vitamin C is being synthesized by the kidneys of the chicken and during heat stress, it becomes insufficient to cope with the bird's requirements. Vitamin C improves immunity, body weight, anti-oxidative potential, semen quality, and carcass characteristics in chicks (Abidin and Khatoon, 2013). A study was conducted to evaluate the effects of vitamin and mineral premix and vitamin C in broiler chicks during 0-35 days of the growing stage under artificially induced heat stress. The results showed that vitamin and mineral premix in water increased body weight gain and feed intake linearly, and the corticosterone level was reduced in serum in all treatment groups as compared to control (Saiz del Barrio et al., 2020). Similarly in another study, Vitamin C was provided to broiler chicks during the growing stage and the feed intake and body weight gain was improved due to a decrease in metabolic heat in birds as compared to the control group where no vitamin C was provided (Abudabos et al., 2018). In another study, Vitamin A, C, E, capsaicin from red pepper, selenium, and zinc were evaluated in layers kept under a heat stress environment. The supplementation increased weight gain, feed efficiency, and immune organs such as spleen and thymus weights, egg weight, shell thickness, and egg-laying percentage as compared to the control. Red pepper improved egg yolk color and decreased total cholesterol and lipids vice versa to selenium (Malekizadeh et al., 2012).

ALA and GAA supplementation

The α-Lipoic acid (ALA) and guanidinoacetic acid (GAA) are naturally synthesized in body and they are also present in many feed ingredients good antioxidants and if supplemented during heat stress, show positive response on production. In a study, α-Lipoic acid was used in broiler chicks kept at high temperatures and it increased average daily weight gain, feed intake, and volatile fatty acids in treatment groups as compared to the control. Moreover, villus height and villus to crypt depth ratio were improved along with the increase of beneficial microbes in the cecum (Wasti et al., 2021). In laying quails, guanidinoacetic acid (GAA) was supplemented during heat stress conditions and it resulted in increased laying capacity, better egg quality parameters, increased activity of superoxide dismutase, glutathione peroxidase, and reduction of malondialdehyde in serum (Raei et al., 2020).

Chitosan and galacto- oligosaccharides supplementation

In a study, chitosan oligosaccharides at 200 mg/kg were supplemented to broilers kept at high temperatures and it resulted that in the treatment group average daily weight gain, feed efficiency, weights of the immune organs, muscle oxidative enzyme activities were increased along with decreased malondialdehyde and corticosterone content as compared to control (Chang et al., 2020). Galacto-oligosaccharides were used in broilers kept at high temperatures and it resulted in improvement of feed conversion rate and weight gain along with a 5% reduction in mortality in treatment groups as compared to the control group (Slawinska et al., 2020).

Copper and zinc nanoparticles supplementation

In a study, nanoparticles of copper oxide were used to decrease heat stress effects on broiler chicks. The results showed that there was a reduction in metabolic heat and an increment in body weight gains in copper nanoparticles supplemented groups. Moreover, the stress biomarker, malondialdehyde (MDA) was decreased along with increased activities of catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase. Moreover, the liver tissue histology parameters were improved along with modulation of heat shock protein 70 and heat shock protein 90 (El-Kassas et al., 2019). In another study nanoparticles of zinc oxide were fed to layers as 80 mg/kg under heat stress and it resulted in a significant increase in egg numbers, egg weight, eggshell thickness, superoxide dismutase (SOD) activity, and zinc bioavailability in the treatment group as compared to control (Abedini et al., 2018).

Zinc, chromium, selenium, and methionine chelates supplementation

Effects of supplementation of zinc, chromium, and selenium in feeds were recorded in broiler chicks during the growing stage under heat stress. The results showed that there was an increase in feed intake and body weight gain in Zn, Se and Cr supplemented groups along with a reduction in lipid peroxidation as compared to the control. Moreover, the activity of superoxide dismutase was increased (Rao et al., 2016). Similarly, Zn bound to methionine and zinc oxide was supplemented to broilers under heat stress and it increased weight gain, nutrient bioavailability, thymus and bursa weights, egg quality, and reduction of malondialdehyde (MDA) and cholesterol in serum (Abd El-Hack et al., 2018). Similarly in another study, Zn-methionine (20, 40, and 80 mg/ kg of feed) provision to layers under heat stress has resulted in increased production, immunity, and egg quality parameters and reduced lipid peroxidation as compared to inorganic Zn and control group (Min et al., 2018). Chromium is well-thought-out to be a valuable trace element. It has been stated that its supplementation encourages the productivity of broilers during heat stress. In a study, chromium was

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supplemented methionine in broilers and the results showed an increase in body weight gain, feed conversion ratio along with the coefficient of energy metabolism, serum glucose, cholesterol, and triacylglycerol (Santos Dalólio et al., 2021). Amino acid-bonded trace minerals were provided to broilers under heat stress and it resulted in a decrease of the corticosterone levels and cytokines in serum and resultantly improved productivity of chicks (Baxter et al., 2020). In another study, ethoxyquin and mineral-methionine chelate were used in broilers and it resulted in a reduction of development of wooden breasts in broilers and the lipid peroxidation of muscles was significantly lower as compared to control when both were used together (Kuttappan et al., 2021).

$NaHCO_3$ and KCL supplementation

Dietary supplementation of $NaHCO_3$ was performed in layers and immune response was improved against ND virus after vaccination, and digestibility of nutrients was significantly higher in treatment groups as compared to the control (Abbas et al., 2019). Potassium chloride was used in broiler chicks under heat stress and it increased the digestibility coefficients of dry matter, protein, fat, fiber, and crude ash. Moreover, the bioavailability of calcium and phosphorous was more for potassium chloride supplemented chicks as compared to the control. The red blood cells and total hematocrit were more in treatment groups as compared to the control (Gorlov et al., 2020).

Propolis supplementation

Propolis is obtained from honeybees and it has several beneficial effects under stressful environments. In a study on quails kept under high temperatures, propolis was used for minimizing the adverse effects of heat stress. It resulted in improvement of weight gain and feed intake, increases in intestinal crypts depth, and immune organ weights (Mehaisen et al., 2017).

Phytogenic supplementation

Many phytogenic feed supplements are being used nowadays to combat stress in poultry birds. Phytogenic feed additives have medicinal properties and natural to use without any side effects. They also enhance feed palatability with the flavor, taste and texture that the birds love to eat. Phytogenics enhance feed intake, promote gastrointestinal health status of the birds, improve immune system, reduce cellular oxidations, and improve production and product quality. There are many phytogenics that are mentioned below.

Ginseng

In a study, ginseng was used in broilers and it resulted in lowering the gene expression of HSPA1A, HSP1, and HSP-16.2, and it was reported to be the most suitable additive to reduce heat stress undesired effects (Sandner et al., 2020).

Dried plums

Dried plums were used in a study on broilers to reduce the harmful effects of heat stress. It increased the daily weight gain, feed intake, and feed conversion rate of the broilers, and it increased the expression of the genes related to anti-oxidation, heat shock proteins, and immune system activation genes as compared to the control (Wasti et al., 2021).

Watermelon juice

Watermelon juice was added as 20% and 40% to water and it was used during the finishing stage of the broilers for 21 days under heat stress. The results showed that the survival rate was improved and it was 100% in the 40% supplemented group. Moreover, weight gain and feed intake were increased significantly in all treatment groups as compared to the control (Jimoh et al., 2020).

Curcumin

Curcumin is obtained from turmeric and it was used in laying hens to improve the immunity during heat stress and it resulted in a reduction of Interleukins-6, interleukins 1β, and tumor necrotic factor- α in treatment groups as compared to the control (Nawab et al., 2019). In another study, curcumin supplementation in broilers at 100 mg/kg of the diet (Zhang et al., 2018) and laying hens at 150 mg/kg of the diet (Liu et al., 2020) has increased growth performance and anti-oxidative and immune regulating gene activities, and egg and meat quality. In another study, curcumin nanoparticles were used in broilers and they were provided with 25, 50, and 100 mg/kg of the diet under heat stress. The results showed a significant increase in body weight gain, feed intake, immune organ weights reduction in malondialdehyde, and an increase in glutathione peroxidase and superoxide dismutase activity. Curcumin nanoparticles at 50 and 100 mg/kg of diet showed more significant results as compared to other groups (Badran, 2020). Similarly, cumin seed oil was supplemented to layers as 500 g/ ton of feed and it resulted in increased egg quality, feed conversion, immunity, and drop in serum malondialdehyde level (Saleh et al., 2019).

Cumin seeds

Cumin seed powder supplementation in broilers under heat stress has resulted in increased weight gain, feed conversion ratio and dressing percentage (Chawke et al., 2021). In a study, the effects of phytogenic feed additive

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"comfort" (PFA-C) was evaluated in broilers and it resulted in down-regulation of heat shock protein 70 gene expression, increase in water and feed intake, and average weight gain and low mortality (Greene et al., 2021).

Cinnamon and clove

In a study, cinnamon and clove powders and their oils were used in quails reared under heat stress and it resulted in a significant drop in heat shock protein HSP70, HSP40, and HSP90, and tissue malondialdehyde, corticosterone, and cytokines in serum were reduced along with the increased activity of catalase, superoxide dismutase and glutathione peroxidase in treatment groups as compared to the control. Moreover, the oils were more beneficial as compared to the powders (Mustafa and Wasman, 2020). Similarly in broilers, cinnamon powder as 0.5% has increased the weight gain and activity of serum glutathione peroxidase, catalase, and superoxide dismutase enzymes and reduction in malondialdehyde content (Sadeghi and Moghaddam, 2018).

Siam weed

Siam weed (*Chromolaena odorata*) was used in broiler chicks to decrease the heat stress effects. Its Ethanol extract at 25–400 µg/ml presented a resilient antioxidant activity in vitro, comparable to 10–80 µg/ml ascorbic acid supplementation. Chromomoric acid obtained from siam weed (*Chromolaena odorata)* at 10 µg has anti-inflammatory activity. Moreover, the toxicity trials have shown that only the aerial fragments of it are nontoxic for animal feeding (Lartey et al., 2020).

Fennel seeds

A study was performed in laying hens where fennel (*Foeniculum vulgare*) at 0, 10, and 20 g/kg were used to decrease harmful effects of heat stress and it resulted that malondialdehyde and egg carbonyl contents were reduced along with egg triglyceride and yolk cholesterol contents (Gharaghani et al., 2015).

Herbal mixture

In a study, extracts of *Phyllanthus emblica, Tribulus terrestris, Withania somnifera, Ocimum sanctum Asparagas racemosus, Mangifera indica,* and *Glycrrhiza glabra* were mixed and used in broilers under heat stress and it resulted in a significant increase in body weight and feed intake. Moreover, free radical formation, blood urea nitrogen, serum cholesterol, and glucose level were decreased significantly in treatment groups as compared to control at 42 days of age (Rao et al., 2021).

African moringa

African moringa (*Moringa stenopetala)* leaf meal was used in broilers and layers kept in a high-temperature environment and it resulted that 2% supplementation of it improved egg yolk color and there was no significant effect on egg and meat quality (Tamiru et al., 2020).

Lycopene

Lycopene is a carotenoid that is being found in tomatoes (*Solanum lycopersicum*) and it has shown anti-oxidative, anti-inflammatory activities along with improved productivity and feed efficiency, and better broiler meat and egg quality (Arain et al., 2017). Similarly in another study in laying hens, lycopene as 20 mg/kg of the diet has shown a decrease in lipid peroxidation and increase in egg yolk color along with mineral and vitamin levels (An et al., 2019).

Licorice

In a study, Licorice (*Glycyrrhiza glabra*) was used in egg-laying quails under heat stress and it resulted in a reduction of egg yolk cholesterol and total lipids in licorice fed groups at 500 mg as compared to the control. Moreover, total serum cholesterol, low-density lipoproteins, triglycerides, and total serum lipids were decreased significantly in treatment groups as compared to control (Dosoky et al., 2021).

Willow

Willow (*Salix*) bark has been used in poultry birds to alleviate the negative impact of heat stress on birds' health and production because of the important source of flavonoids, tannins, salicin, and glucosides of saligenin (Saracila et al., 2021). Organic acids and essential oils are safe and have been approved by European Union for supplementation in animal feeding as anti-oxidative, antimicrobial, immune stimulators, and stress relieving additives (Doneria et al., 2020).

Syrian oregano

In a study, Syrian oregano (*Origanum syriacum*) oil and Avilamycin, an antibiotic, effects were compared in broilers under heat stress for 42 days and the results showed no significant difference between the effects of the 600 mg/kg *Origanum syricaum* and avilamycin as compared to the control and it has shown improvement in production and immune status of the birds under heat stress (Tekce et al., 2021).

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Garlic

Individual and combined effects of probiotic, garlic powder, citric acid was evaluated in broiler chicks under chronic heat stress. It resulted in improvement of productivity and feed efficiency, immunity, intestinal microbial population, and intestinal morphometric characters in treatment groups as compared to the control (Elbaz et al., 2021).

Thyme

In a study on rabbits, thyme leave powder was used for 90 days to minimize the deleterious effects of heat stress and it resulted in a significant increase in the feed intake, body weight gain, liver and kidney functionality, improved testosterone and semen parameters in all treatment groups as compared to the control and particularly for 16 g/kg diet supplementation of the thyme leave powder the results were highest for all the above parameters (Ahmed et al., 2020). In another study, thyme oil as 150, 200 mg/kg of diet in broilers under heat stress has resulted in a significant increase in weight gains, immunity, and reduced oxidative stress biomarkers (Khafar et al., 2019).

Onion

Onion powder as 2.5 kg/ton of feed and 2.5% onion extract in drinking water was used for broiler chicks under heat stress for 42 days and the results showed an increase in production and feed intake of the chicks, immunity, and heat tolerance as compared to control (Al-Ramamneh, 2018).

Grade seeds

In a study on the broiler, grape seed extract and vitamin C was supplemented for 42 days to cope with chronic heat stress and it resulted that 300 mg/kg grape seed extract caused a significant increase in body weight gain, decrease glucose, cholesterol, triglycerides and low-density lipoproteins in serum of the chicks of treatment groups as compared to the control (Hajati et al., 2015).

Saffron

Saffron (*Crocus sativus*) petal extract 300, 500, and 700 mg/kg of diet was used in broiler chicks for 42 days under heat stress and it resulted in a significant increase in feed intake and body weight gain, glutathione peroxidase, and superoxide dismutase activities and significant decrease of serum cholesterol, uric acid and malondialdehyde in treatment groups as compared to the control (Hosseini-Vashan and Piray, 2021).

Sweet wormwood

Sweet wormwood (*Artemisia annua*) was used in a study on broilers under heat stress to evaluate its antioxidant potential and relative meat quality parameters and it resulted in a significant increase of redness and energy status of breast muscles and decreased drip loss, reactive oxygen species production and the recommended level of use was 1-1.25 g/kg of diet (Wan et al., 2018).

Tulsi leaf and fenugreek seed

In a study, tulsi (*Ocimum sanctum*) leaf powder and fenugreek (*Trigonella foenum-graecum*) seed powder was used in broilers and the results showed that under a high level of temperature during experimentation the feed cost was not significantly changed but the production was improved in treatment groups as compared to the control (Prajapat et al., 2020).

Resveratrol (Red grapes)

Resveratrol obtained from red grapes was supplemented to layers under heat stress at 0, 200, 400, and 600 mg/kg of the feed and it resulted in improved productivity and humoral immunity and reduced serum malondialdehyde and gene expression of heat shock proteins (Liu et al., 2014). Similarly, ducks were supplemented with resveratrol under acute heat stress exposure and it resulted in increased villus heath to crypt depth ratio, a higher number of goblet cells, increased tight junction proteins and reduced gastrointestinal artifacts, reduced gene expression of heat shock proteins (Yang et al., 2021). Similarly in another study on broilers, resveratrol supplementation has shown an increase in activity of superoxide dismutase and glutathione peroxidase and reduction in serum and meat malondialdehyde (He et al., 2019).

Green tea

Catechin extracted from green tea was supplemented to broilers under heat stress as 300 and 600 mg/kg of the feed and it resulted in a significant increase in weight gain and feed intake, enzymatic activities and immune response, and reduction in serum cholesterol as compared to control (Xue et al., 2017). Similarly in quails under heat stress catechin supplementation has resulted in increased feed intake and egg numbers and better antioxidant profile (Sahin et al., 2010).

Black cumin seeds

Black seeds (*Nigella sativa*) supplementation as 1-2% in broilers has increased weight gain and meat quality, reduced oxidation, and serum corticosterone levels (El-Shoukary et al., 2014).

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Rosemary

Rosemary powder supplementation as 0.4% in broilers under heat stress has shown an increase in weight gain and feed intake (Petričević et al., 2018). Similarly, rosemary extracts in broilers under heat stress have resulted in improved weight gains, reduced serum malondialdehyde, and decreased gene expression for heat shock proteins (Tang et al., 2018). Similarly, many studies have been done and showed that dietary manipulations are very beneficial in poultry production.

CONCLUSION

Due to environmental risks and global warming, heat stress is the prevailing menace to the poultry industry which should be handled to prevent economic loss and improve bird welfare. Management strategies are not sufficient to decrease deleterious effects. Therefore, for optimal upgrading of poultry production under stressful conditions and improving their health and immunity valuable nutritional adaptation should be used. Dietary management can be actual in incapacitating the harmful influences of heat stress and can increase production and welfare in terms of improved broilers weight gains and egg-laying capacity, better egg and meat quality, reduce oxidative stress, and enhance immunity. Nevertheless, more studies are required to explore the mutual outcomes of more or less of the above-mentioned nutritional plans, either single-handedly or in combination with management strategies, to decrease the damaging effects of heat stress and to observe their efficiency and economical value in poultry husbandry.

DECLARATIONS

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Authors' contribution

Syed Rizwan Ali contributed for the write up of the study design and the final manuscript. Ibrahim Sadi critically revised the manuscript for important academic contents.

Conflict of interests

The authors have not declared any conflict of interests.

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DEVELOPMENT OF A REAL-TIME PCR ASSAY FOR THE RAPID DETECTION OF *netB* GENE IN *CLOSTRIDIUM PERFRINGENS*

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ABSTRACT: Necrotic enteritis (NE) has a huge economic impact in the poultry industry. Numerous studies have shown that necrotic enteritis toxin B (*netB*) is a key virulence factor in *Clostridium perfringens* strains that cause NE in chickens. Early detection of *netB* in *C. perfringens* is important to circumvent the spread of NE. In this study, we developed a novel combinatorial approach involving (i) a commercial 2-step DNA extraction kit and (ii) a real-time polymerase chain reaction (qPCR) for the detection of *netB*-positive *C. perfringens* in fecal samples. Melt curve analysis and specificity test demonstrated 100% specificity without any cross-reactivity in other bacterial species with a limit of detection of 10^2 cfu/g. Field validation was subsequently conducted on nine fecal composites collected from different layer houses at two commercial farms, leading to successful detection of four *netB* positive samples. The study presented a rapid diagnostic qPCR assay involving a 2-step DNA extraction protocol to screen for *C. perfringens* carrying *netB* gene in chicken fecal samples.

Keywords: *Clostridium perfringens;* Necrotic enteritis; *netB* gene; Poultry; Real-time PCR

INTRODUCTION

Necrotic enteritis (NE), an important enteric disease, has a huge economic impact on the poultry industry. While chronic cases typically result in a loss of productivity due to poor feed conversion ratio, a significant level of chicken mortality could be observed in acute cases (Lovland and Kaldhusdal, 2001). It is projected that NE in chickens can cost the global poultry production industry to an estimated of US$3 billion losses annually (Mcdevitt et al., 2006).

Although many predisposing factors play a role in the pathogenesis of the disease, the main causative agent for the dissemination of NE is the Gram-positive *Clostridium perfringens*. Toxicogenic classification of *C. perfringens* (Class A-E) is based on the production of four major toxin variants (alpha, beta, epsilon, and iota). It is well documented that NE is typically caused by the Class A isolates (Ezatkhah et al., 2016; Merati et al., 2017). Historically, a phospholipase C enzyme, alpha toxin, was long thought to be the important virulence factor in NE (Keyburn et al., 2008). However, the role of a novel beta-pore forming toxin, necrotic enteritis toxin B (*netB*), has been identified as a major pathogenicity factor in NE (Keyburn et al., 2008). A seminal study conducted by Keyburn et al. (2008) demonstrated that an alpha-toxin null mutant of a virulent NE isolate can still cause disease in chicken, signifying the non-essential role of alpha-toxin in NE. On the contrary, *C. perfringens* with mutated *netB* gene were avirulent while the pathogenicity of complemented strains was fully restored, demonstrating the critical role of *netB* in causing NE (Keyburn et al., 2008; Rood et al., 2016).

Since the discovery of *netB*, several studies have initiated the screening of the gene within a wide range of *C. perfringens* isolates. For instance, initial screening of a range of Australian poultry NE isolates found that majority of the strains (70%) were *netB* positive (Keyburn et al., 2010). In a separate study, *netB* was found in most of the *C. perfringens* isolated from chickens displaying clinical signs of necrotic enteritis (Rood et al., 2016). A study conducted in Canada by Chalmers et al. (2007) also showed that *netB* gene was predominantly detected in animals associated with NE. Similarly, Mwangi et al. (2019) also reported that 81% of the diseased chickens were *netB* positive.

To circumvent the spread of NE and better inform the stakeholders on the health status of the livestock, the ability to detect the presence of *netB* toxin is critical. For example, Lee et al. (2021) first reported the use of *netB*-specific mAb-based technique to determine the presence of native *netB* toxin in biological samples from NE-infected chickens.

Since the conventional polymerase chain reaction (PCR) was developed in 1983 for detection of DNA, the realm of molecular biology has been at the forefront for microbial diagnosis (Kralik and Ricchi, 2017). Compared to culture-based assays which typically require few days to completion, PCR-based detection method can be done rapidly with a higher sensitivity. An advancement from the conventional PCR technique, particularly quantitative PCR (qPCR), is often considered a method of choice for detection of microorganisms (Rinttilä et al., 2004). Given the high prevalence of *C. perfringens* in fecal samples (Hustá et al., 2020), this study embraced two main objectives; first, we developed a real-

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time qPCR probe-based assay that detects *netB* positive isolates from fecal samples; second, we also evaluated a commercial kit consisting of a 2-step protocol to concomitantly (i) extract genomic DNA from fecal samples and (ii) stabilize the DNA at room temperature post-extraction. This allows ease of transportation of the extracted DNA to a laboratory to conduct qPCR without the need for a cold chain facility. Furthermore, we applied this assay for selective detection of self-spiked *netB* positive *C. perfringens* in fecal samples and deployed this assay to two commercial layer farms for on-field evaluation.

MATERIALS AND METHODS

Ethical Approval

Approval to carry out this study was not required as no invasive method was involved.

Detection of *netB* gene toxin from *Clostridium perfringens* field isolates

Six *Clostridium perfringens* field isolates were screened for *netB* using conventional PCR with primers from Keyburn et al. (2008) (Table 1). Confirmatory tests such as iron-milk presumptive test, motility-nitrate test, and lactose-gelatin test were carried out for identification of the isolates. Briefly, DNA extraction of a single *C. perfringens* colony was carried out by resuspending the colony in 50 µL of sterile distilled water and heating the suspension at 98 °C for 10 minutes. The PCR amplification was conducted in a 25 µl volume consisting of 2 µl of DNA template, 0.3 µM of primers, 1x DreamTaq™ Green PCR Mastermix (Thermo Fisher Scientific™, USA) and Water, nuclease free (Thermo Fisher Scientific™, USA) on a thermocycler (miniPCR®, USA). The PCR conditions involved initial denaturation for 120 seconds at 95 °C, followed by 35 cycles of DNA denaturation for 15 seconds at 95 °C, annealing at 55 °C for 30 seconds, and extension at 72 °C for 30 seconds, with an additional extension for 5 minutes at 72 °C at the end of the 35th cycle. For amplicon visualization, 18 µl of amplified fragments were premixed with 2 µl of 10x BlueJuice™ Gel Loading Buffer (Invitrogen™, USA) and loaded into the wells of agarose gel containing SYBR™ Safe DNA Gel Stain (Invitrogen™, USA). Amplicons were observed using an electrophoresis visualization system, blueGel™ (miniPCR®, USA), with BenchTop 1 Kb DNA Ladder (Promega™, USA) for amplicon size estimation. The *netB* positive isolates were cryopreserved for subsequent downstream real-time PCR assay development.

Table 1 – Primers and probe used in conventional and real-time PCR for detection of *netB* gene

Primer or Probe	Sequence (5' – 3')	Size (bp)	Reference
AKP78 (Forward)[1]	CTTCTAGTGATACCGCTTCAC	383	Keyburn et al. (2008)
AKP79 (Reverse)[1]	CGTTATATTCACTTGTTGACGAAAG		
netB Fwd (Forward)[2]	TAATGGTGATAAAAATTTCACAGAT		
netB Rev (Reverse)[2]	TTAGCATTTTTAGGTGCTGTTA	105	-
netB Probe[2]	CTGGTGGATTTTCACCCAATATGGCTTTAG (FAM)		

[1]: Primer / Probe used in conventional PCR assay; [2]: Real-time PCR assay

Development of real-time PCR (qPCR) for detection of *netB* gene in *Clostridium perfringens*

Primer pair netB Fwd and netB Rev targeting 105 bp of the *netB* gene was designed using Primer3Plus (Untergasser et al., 2012) as shown in Table 1. The primer pair was designed using *Clostridium perfringens* strain EHE-NE18 plasmid pJIR3535 as the reference *netB* gene (Accession: CP025502.1). Basic Local Alignment Search Tool (BLAST®) was carried out to ensure homology of the primers to the *netB* gene sequence (Altschul et al., 1990). A temperature gradient was carried out to determine the optimal annealing temperature (55 °C) of the primer pair. To ensure the specificity of the primer pair, a fluorescent melt curve analysis was also performed. Synthetic *netB* fragment (Integrated DNA Technologies™, Singapore) was used as a positive control. The length of the fragment was 419 bp with coverage of nucleotide position from 30215 to 30633 of plasmid pJIR3535. A 20 µL reaction mixture containing synthetic *netB* DNA fragment, 0.3 µM of Fwd/Rev primers, 1x SensiFAST™ SYBR™ No-ROX Mix (Meridian Bioscience®, USA) and PCR Grade water was analyzed using Azure Cielo™ 3 (Azure Biosystems™, USA). The qPCR cycle used in this assay was as follows: Initial denaturation for 180 seconds at 95 °C, followed by 45 cycles of DNA denaturation for 5 seconds at 95 °C, annealing of primers and extension of template at 55 °C for 15 seconds.

Real-time TaqMan™ FAM probe targeting *netB* gene was designed using an online tool, Primer3Plus. Similarly, BLAST alignment was carried out to ensure homology of the probe designed. A 20 µL qPCR reaction mixture consisting of template, 0.3 µM of Fwd/Rev primer, 0.2 µM of probe (FAM), 1x SensiFAST No-ROX Probe Mix and PCR Grade water was analyzed using Azure Cielo™ 3 (Azure Biosystems™, USA). A total of three technical replicates along with corresponding positive/negative controls were included in the analysis.

The *netB* specificity test was further analyzed empirically. Common inhabitants found in the gut such as *Salmonella* spp*, Clostridium difficile, Clostridium butyricum, Campylobacter jejuni, Lactobacillus fermentum, Streptococcus suis, Enterococcus faecalis, Escherichia coli, and Bacillus subtilis* were tested for non-specific binding of *netB* primer pair and probe (Table 1). A *netB* negative *C. perfringens* ATCC® 13124 was also included in the analysis for potential cross-reactivity within the same species.

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Detection of *netB* positive *Clostridium perfringens* in fecal samples

Clostridium perfringens isolate carrying *netB* gene (M4.6) was cultured at 37 °C in Thioglycollate Medium USP (Oxoid™, UK) for 18 h. A ten-fold serial dilution was performed from the overnight culture to prepare a series of standards for spiking. The bacterial standards were plated on Perfringens Agar (Oxoid, UK) to quantify the bacterial load. Chicken fecal samples (4 g) were artificially spiked with the standards to a final concentration ranging from 10^1 to 10^6 cfu/g. Arcis Sample Prep Kit (Arcis Biotechnology, UK) was used for DNA extraction. Briefly, Reagent 1 (R1) containing lysis buffer and DNA stabilizer to rapidly disrupt cell membranes and protect the released DNA from degradation was added. Prior to qPCR process, an equal volume of Reagent 2 (R2) containing wash buffer was added to the extract. DNA sample was further purified from the R2-treated fecal samples using QIAamp® DNA Mini Kit (Qiagen®, Germany) according to the manufacturer's protocol. Prior to the qPCR, an exogenous internal control (Primerdesign, UK) was added to the reaction mix to check for potential PCR inhibition. In brief, the 20 µL qPCR reaction mixture contained template (sample and internal control), 0.3 µM of Fwd/Rev primer, 0.2 µM of probe (FAM), 1x internal control primer/probe mix (VIC), 1x SensiFAST No-ROX Probe Mix and PCR grade water.

Detection of naturally occurring *netB* positive *Clostridium perfringens* isolates in fecal samples

Validation study was conducted to evaluate the efficacy of this assay under field condition. Fecal samples were collected from two independent layer farms with a closed house system. A total of 50 g fecal composite was randomly collected from 3 collection points (front, middle and back) of the house. In this validation trial, a total of nine fecal composites were evaluated. Each of the fecal composite was homogenized using a disposable spatula, and 20 g of the composite was subsequently transferred to a sterile stomacher bag. An 80 ml of sterile Phosphate Buffered Saline was added to the stomacher bag and mixed homogenously. The content of the bag was left to stand for 10 minutes to allow sedimentation of debris particles. A 180 µL of the supernatant was added into a sterile microcentrifuge tube containing 300 µL of R1 (Arcis Biotechnology, UK). The stabilized extractants were delivered to a laboratory for qPCR analyses. Prior to qPCR process, an equal volume of R2 containing wash buffer was added to the fecal extract to remove nucleic acid chelation and enhance PCR reaction. QIAamp DNA Mini Kit was used subsequently for further purification and concentration of DNA according to the manufacturer's protocol. Real-time PCR analysis was then carried out for the purified samples.

RESULTS

Detection of *netB* gene toxin from *Clostridium perfringens* field isolates

Six *Clostridium perfringens* field isolates were cultured at 37°C in Thioglycollate Medium USP (Oxoid, UK) for 18 h. Subsequently, the cultures were plated on Perfringens OPSP Agar Base (Oxoid, UK) supplemented with Selective Supplements A and B (Oxoid, UK). The identity of all the field isolates was identified as *C. perfringens* through series of confirmatory tests. A conventional PCR amplification was subsequently deployed to screen for the presence of *netB* in *C. perfringens*. In this screening test, an expected *netB* amplicon size of 383 bp was observed for isolates S5.1 and M4.6 (Figure 1). On the other hand, *netB* was not detected for isolates S5.2, S5.3, M4.1, M4.3, and no template control (NTC) in the PCR assay. Isolates S5.1 and M4.6 were subsequently cryopreserved for subsequent downstream qPCR development.

Melt curve analysis

The melting curve analysis resulted in a single peak with an average melting temperature (Tm) of 76.6 °C to 77.2 °C. As shown in Figure 2, melting temperature of isolate S5.1 and M4.6 were close to the synthetic *netB* fragment, demonstrating the specificity of the primer pairs. No peaks were observed in the no template control (NTC).

Limit of detection (*netB* positive *Clostridium perfringens* isolate)

Limit of detection was determined as the lowest concentration of the isolate that can be detected using this assay. The range of detection spanned from 10^2 cfu/g to 10^6 cfu/g of *netB* positive *Clostridium perfringens* isolate. No significant difference was observed for the Cq value of the internal control for all the dilutions evaluated. As the assay was not able to detect fecal sample spiked at 10^1 cfu/g, the limit of detection of this assay was determined to be 10^2 cfu/g.

Specificity test

Specificity for *netB* real-time PCR assay was 100%. Expectedly, this assay was able to detect *Clostridium perfringens* isolate (S5.1) carrying *netB* with Cq value of 32.53. No cross reactivity was observed for other microorganisms tested, including *netB* negative *C. perfringens* ATCC 13124 strain in this test.

Field validation

Amongst the nine composites collected from different layer houses at two commercial farms (farm A and B), four of the samples were detected positive for *netB* gene (Table 3). An exogenous internal control was added into the reaction mixture to distinguish true target negatives from false negatives arising from potential PCR inhibition. As shown in Table 3, the Cq (VIC) values of the internal control across six samples were within the expected range of 28 ± 3, signifying no PCR inhibition in the test run.

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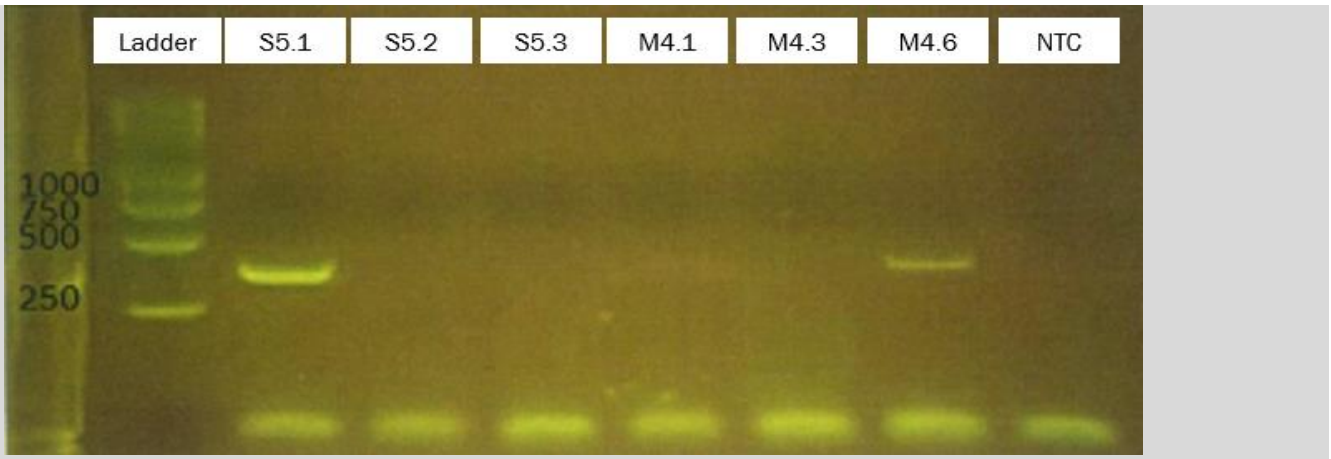

Figure 1 – Gel electrophoresis image of *Clostridium perfringens* isolates screening.

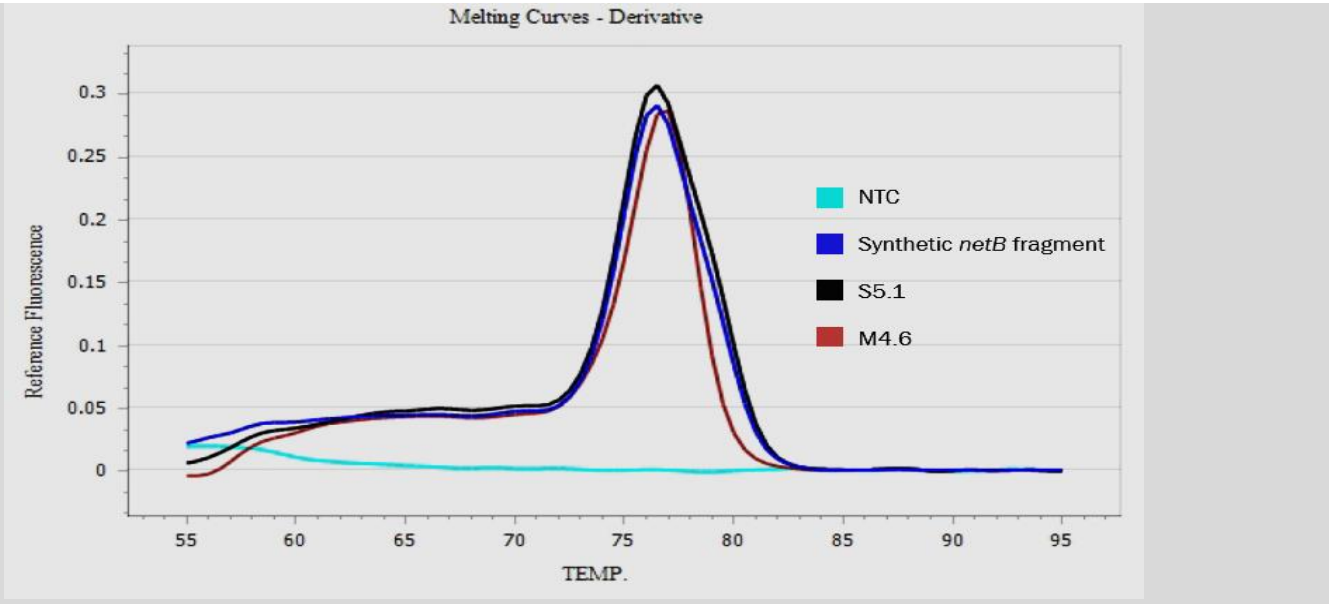

Figure 2 – Melt curve analysis of *netB* amplicons from real-time PCR.

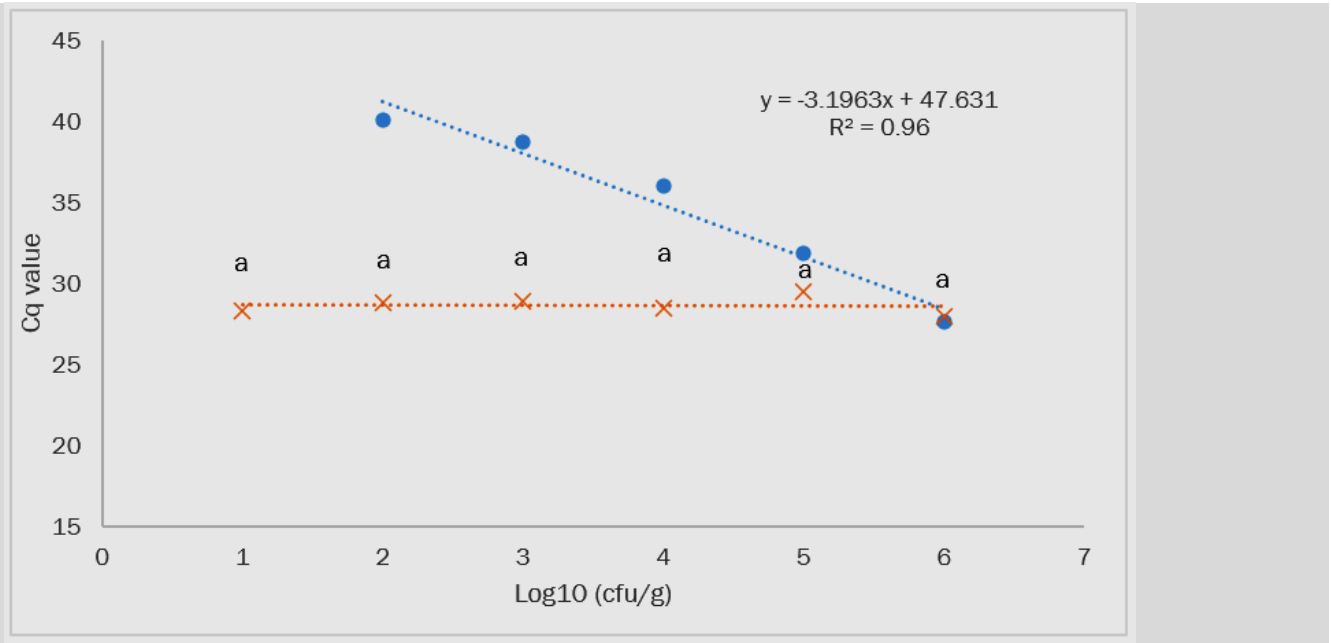

Figure 3 – Standard curve generated from fecal samples spiked with different levels of *netB* positive *Clostridium perfringens* standards (●). Cq values of internal control (×) were plotted to check for level of inhibition. At each dilution, the Cq value was plotted against the logarithm of the concentration of amplicon (cfu/g). Cq values of the internal control were not statistically different between the different dilutions ($p>0.05$) using One-Way ANOVA.

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Table 2 – Real-time PCR results on the specificity of the primer pair and probe on difference bacteria.

Bacteria	Strain	Cq (FAM)
Clostridium perfringens[1]	S5.1	32.53
Clostridium perfringens	ATCC® 13124	-
Salmonella spp.[2]	Feed Isolate	-
Clostridium difficile	ATCC® 9689	-
Clostridium butyricum	ATCC® 19398	-
Campylobacter jejuni	ATCC® 35918	-
Lactobacillus fermentum	ATCC® 23271	-
Streptococcus suis	ATCC® 99849	-
Enterococcus faecalis	NUS-EL 7/10 P4	-
Escherichia coli	ATCC® 25922	-
Bacillus subtilis	PB6	-

[1] *Clostridium perfringens* S5.1 was used as a positive control. [2] *Salmonella* spp. was isolated from feed sample and identified using Analytical Profile Index (API 20) E.

Table 3 – Field validation results of fecal composite samples from Farm A.

Sample	Cq (FAM)	Cq (VIC)
House 1	-	26.68
House 2	-	27.07
House 3	-	27.16
House 4	-	26.77
House 5	35.96	26.79
House 6	36.72	26.73
Positive Control	32.93	26.65
Negative Control	-	27.17
NTC	-	-

Table 4 – Field validation results of fecal composite samples from Farm B.

Sample	Cq (FAM)	Cq (VIC)
House 1	40.23	28.39
House 2	40.36	27.32
House 3	-	28.01
Positive Control	35.01	27.72
Negative Control	-	27.84
NTC	-	-

DISCUSSION

Necrotic enteritis is an important disease in the poultry industry. It has been well documented that the key contributing virulence factor of NE is a pore forming heptameric NetB toxin (Keyburn et al., 2008). In this study, we initially obtained two *netB*-positive *Clostridium perfringens* field strains through conventional PCR screening. The discovery of the bacterium carrying *netB* gene forms the experimental basis to further develop a *netB* real-time PCR diagnostic assay. We first designed a qPCR primer pair targeting 105 bp of the *netB* nucleotide sequence. A BLAST alignment was performed against the publicly available *netB* gene reference to ensure the coverage of the sequence of primers is within the consensus sequence of the target gene. Our melt curve analysis (Figure 2) and specificity test (Table 2) demonstrated the high specificity of our primers and FAM-based probe.

Preserving the integrity of DNA is critical for validation of different analyses, and this undertaking often requires different types of equipment for storage which may not be accessible in field (Groenenboom et al., 2019). To simplify the DNA extraction and storing process at a resource limited setting (e.g. farm), we coupled a simple 2-step DNA extraction process with our pre-established qPCR assay. This 2-step process incorporates two different reagents (R1 and R2) to (i). lyse bacterial cells, (ii) stabilize the genetic elements at field level, and (iii) enhance downstream PCR reaction through removal of inhibitors. With this rapid and convenient extraction method, we envision that any untrained farm personnel with limited resources will be able to collect, composite, and extract the DNA from fecal samples conveniently prior to sending the extracted sample to a scientific laboratory for real-time PCR analyses.

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To validate our combinatorial approach involving the 2-step DNA extraction and qPCR processes, first we artificially spiked layer fecal samples with different *C. perfringens* standards (10^1 to 10^6 cfu/g) to determine the limit of detection (LoD). We showed that our assay can be used to detect *C. perfringens* down to 10^2 cfu/g of bacterial load (Bustin et al., 2009). However, it is worth noting that we were not able to recover *netB* gene in fecal samples spiked with 10^1 cfu/ml of *netB* positive *C. perfringens*. It could be attributed to the high concentration of DNA from background bacteria which may reduce the PCR efficiency for low target load (Forootan et al, 2017; Pan et al., 2017) or sampling error due to low DNA concentration (Wilson, 1997; Tellinghuisen, 2020). At present, we are trying to improve the sensitivity of this assay through a combinatorial approach involving different extraction methods and chemicals.

For actual on-field evaluation, a total of nine fecal composites were collected from two independent layer farms. We utilized the 2-step extraction method involving R1 at initial stage to lyse the bacteria cells and stabilize the released DNA. Following that, the R1-treated samples were delivered to the laboratory for actual qPCR analyses. Four of the fecal composites were detected positive for *netB* (Table 3 and 4). As shown, the Cq value of our internal control across all nine samples were within the recommended range, suggesting no PCR inhibition in our assay. Altogether, our result demonstrated that qPCR assay could be used as a rapid environmental surveillance tool to detect the presence of *netB*-positive *C. perfringens* in fecal samples. Numerous studies have indicated that high load of *C. perfringens* carrying *netB* gene in poultry fecal is a strong indicator of NE occurrence in livestock (Chalmers et al., 2007; Mwangi et al., 2019; Thi et al., 2021). We propose that this real-time PCR assay can be deployed on field to evaluate pathogenicity potential of field isolates through detection of the virulence factor *netB* in *C. perfringens*.

CONCLUSION

The real-time PCR assay is a promising tool that can be deployed in the farm to detect presence of *netB*- positive *Clostridium perfringens* in chicken fecal samples. It can potentially be used as a routine surveillance system to diagnose the flocks' health conveniently. Fundamentally, detection of *netB*-positive *C. perfringens* in fecal samples may suggest potential field circulation of the pathogens amongst the flocks. As the *netB*-targeted approach is highly selective against pathogenic *C. perfringens*, this non-invasive assay could provide an alternative sampling technique for monitoring of potential NE outbreak in the farm segment.

DECLARATIONS

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Authors' contribution

G.H. Soo proposed the design of study, prepared the manuscript, and performed the laboratory analysis. J.W.H. Tan and H.C. Ong assisted in the design of study and reviewed the manuscript.

Conflict of interests

The authors declared that there is no conflict of interest in this study.

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THE IMPACT OF STABLE FLIES (*Stomoxys calcitrans* L.) ON SMALL STOCK PRODUCTION IN BODIBENG, BOTHATOGO AND SEHITHWA IN THE NORTH WEST DISTRICT, BOTSWANA; A SURVEY STUDY

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ABSTRACT: Stable fly, *Stomoxys calcitrans* L. (Diptera: Muscidae) is a globally recognized livestock pest of economic importance, which also attacks wild animals, pets and humans. These flies frequently feed on the forelegs of animals and can cause significant production losses and severe animal health and welfare concerns. This study investigated the impact of stable flies on small stock (sheep and goats) production and documented control measures adopted by farmers in Sehithwa, Bodibeng and Bothatogo villages in the North West District of Botswana. Simple Random Sampling was used to select 90 respondents in the study area. Data on demographic characteristics (i.e., age, sex, marital and educational status of the respondents), control measures against stable flies, time stable flies appeared, factors contributing to abundance of stable flies, and the role of government and private sector in the control of stable flies were collected and analysed using SAS. The Chi-square test of goodness of fit was used to show the unequal distribution of the frequencies of respondents among the categories for each variable. Results showed that wood smoking (53.33%) was the common control measure against stable flies followed by migration to unaffected areas (35.55%) and dipping (11.11%). Feeding activity of stable flies reached its peak in the evening (58.89%) followed by morning (31.11%), afternoon (6.67%) and the least was night (3.33%). Eighty-seven percent of respondents mentioned that stable fly contributed to poverty, starvation (16.67%) and loss of income (13.33%). It is concluded that stable flies affect livestock productivity and people' livelihood; hence the need to adopt effective control measures. Control measures against these flies will be more effective when applied in the evening and morning.

Keywords: Control measures, Economic losses, Livestock pest, Small stock, Stable flies.

INTRODUCTION

Stable flies (*Stomoxys calcitrans* L.) are among the important livestock pests in most parts of the world (Showler and Osbrink, 2015; Taylor et al., 2020; Rochon et al., 2021). Kneeland et al. (2012) reported that stable flies are cosmopolitan pests of livestock, wild animals, pets and humans. They have been reported as pests of livestock and humans since the late 1800s to early 1900s (Cook, 2020). Stable flies are hematophagous insects that feed on fore legs of cattle (Erasmus, 2015). Stable fly is a cosmopolitan biting fly of both economic and welfare concern, mainly due to its painful bite, which can cause blood loss, discomfort and loss of livestock productivity (Parravani et al., 2019). According to Taylor et al. (2020), the painful bites from stable flies induce costly behavioral and physiological stress responses and reduce productivity. The flies ignore swatting, stamping and other tactics that the animals use to try to avoid the bites (Kaufman and Weeks, 2019). Stable fly is commonly known as *lethobo* in the North West district of Botswana where it causes economic losses on livestock following the rainy season. Stable fly parasitism has the greatest effect on the livestock industry where animals are confined to stables or pastures, providing suitable conditions for feeding and oviposition of the flies (Kneeland, 2011). Taylor et al. (2006) reported that the stress from *S. calcitrans* painful bites causes confined animals to bunch together or stand in water in an endeavour to escape the fly annoyance or perform avoidance behavior. This results in a significant decline in weight gain or milk production.

Stable fly is the primary pest of cattle that causes major economic losses estimated to over 1 billion USD in cattle feedlots, cattle dairies and in poultry farms in the United States (Kneeland, 2011). Both female and male flies feed on blood and are persistent feeders that cause significant irritation to their host. They can be distinguished with their distinct stiletto like proboscis that extend forward beyond the head. This sharp point beak is used to pierce the skin and draw blood (Kneeland, 2011). According to Showler and Osbrink (2015), stable flies mostly feed on the lower parts of animal

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front legs where hair is less and shorter. The study by Cruz-Vázquez et al. (2004) in Mexico reported that the population of *S. calcitrans* increases fast when the humidity is high due to seasonal rainfall. This explains why *S. calcitrans* occurs following heavy rains in the North West district of Botswana.

Arable and livestock production are practised in Ngamiland East Agricultural District, however, livestock production forms the main agricultural activity. The district comprises 17 extension areas including Bodibeng, Bothatogo and Chanoga (research sites). Statistics Botswana (2018) estimates the populations of cattle, sheep, goats, horses and donkeys in Ngamiland East to be 94300, 10352, 61332, 3169 and 9288, respectively. Although stable fly causes enormous significant livestock mortalities and decreased productivity in the North West District of Botswana resulting in economic losses, no studies have been conducted on this pest. Therefore, a survey study was conducted to investigate the impact of stable flies on small stock (sheep and goats) production and to document measures adopted by farmers to control this pest in Bodibeng, Bothatogo and Sehithwa villages of the North West district of Botswana.

MATERIALS AND METHODS

Study area

The study was conducted in accordance with ethical regulations (i.e., BUAN-AEC-2022-01) approved by the Animal Ethics Committee of Botswana University of Agriculture and Natural Resources. The North West District which comprises Ngamiland East and West has an estimated human population of 175 631. Ngamiland East and Ngamiland East have human populations of 90 334 and 59 421, respectively (Buthali, 2014). The study was conducted in Bodibeng, Sehithwa and Bothatogo of Ngamiland East in the North West District of Botswana in February 2021. The location coordinates for each village are as follows: Bodibeng 20 37'22.79"S and 22 36'6. 59"E; Sehithwa 20 28'00"S and 22 43'00 and Bothatogo -20 47'68.8"S and 22 71 '36.2"E (Statistics Botswana, 2015). The estimated human population for Sehithwa is 2748, 778 for Bodibeng and 555 for Bothatogo (Statistics Botswana, 2015). The distance of the villages from Maun (the district's capital) is as follows: Sehithwa 100 km, Bodibeng 136 km and 112 km for Bothatogo. The research sites (i.e., Bodibeng, Bothatogo and Sehithwa) are shown in Figure 1.

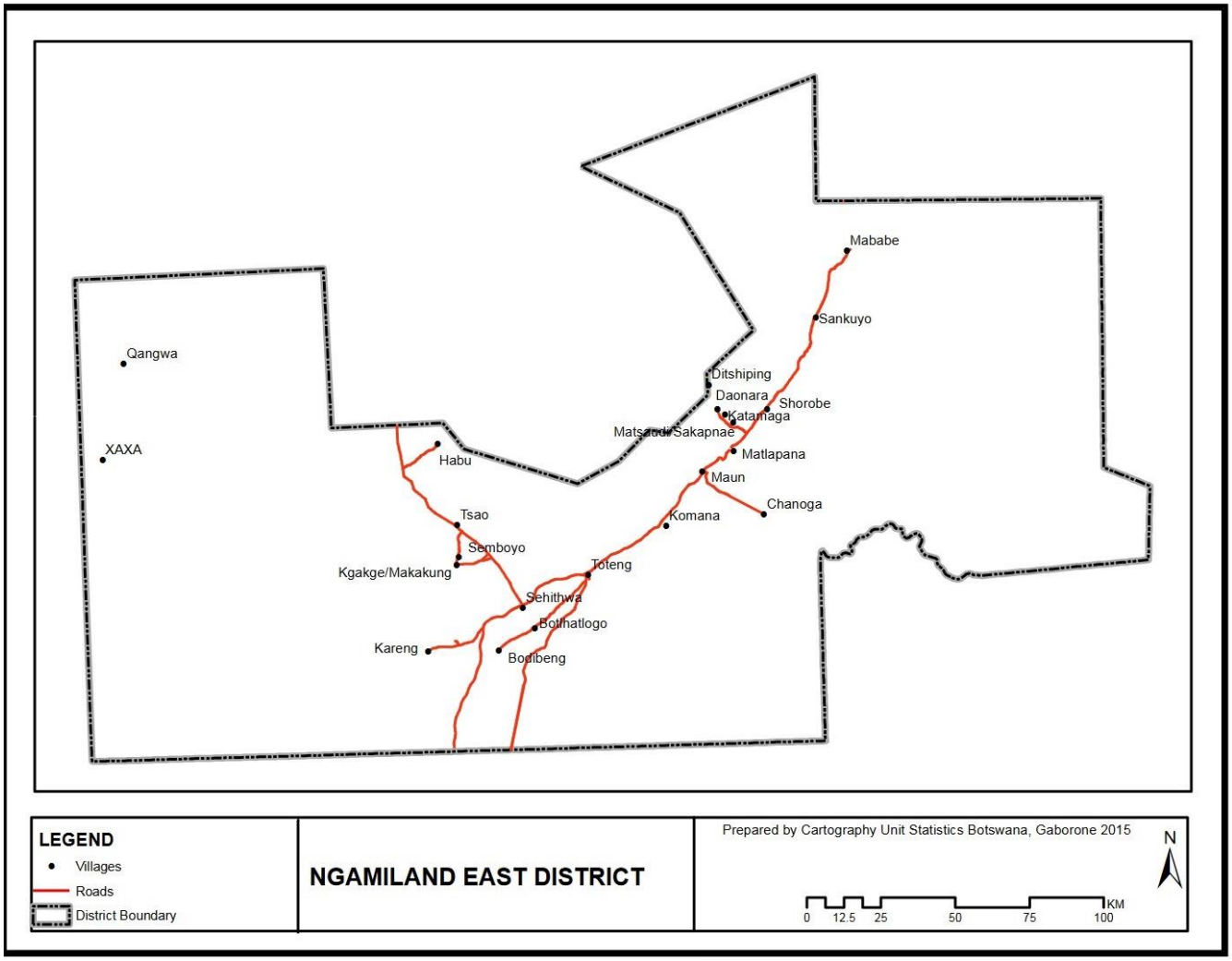

Figure 1 - Map of Ngamiland East District showing the research sites (Source link)

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Sample design

Simple Random Sampling technique was used to select 90 respondents in the study area (i.e., Bodibeng, Bothatogo and Sehithwa). Sample size was based on the population size of the research sites/villages (Table 1).

Table 1 - Sample size of research sites

Village	Estimated population	Sample size
Sehithwa	2748	40
Bodibeng	778	30
Bothatogo	555	20
Total	3581	90

Sources: Statistics Botswana (2015)

Data collection

Data were collected using a structured questionnaire that was administered to 90 respondents across the three villages to gather information on stable flies and their impact on small stock production. Data were also collected from secondary resources. Interviews were conducted in local languages (i.e., Setswana and Herero) and responses written in English. Respondents were asked to give information on sex, educational level, age, practices used to control stable flies, time stable flies appears, factors that contribute to the abundance of stable flies, as well as, role of Government and private sector in the control of stable flies in the study area.

Data analysis

Data derived from questionnaires were captured into a spread sheet and analysed using Statistical Analysis System (SAS, version 9.4) (SAS Institute, 2002-2012). Data were subjected to frequency analysis. Summary statistics are presented in tables and figures. The Chi-square test of goodness of fit was used to show the unequal distribution of the frequencies of respondents among the categories for each variable.

RESULTS AND DISCUSSION

Demographic characteristics of the respondents

Data on demographic characteristics of the respondents are presented in Table 2. The majority (54.44%) of the respondents were married followed by singles (38.89%) with the least being divorced (6.67%). About 51% of the respondents were females and the remainder males. In this study, majority of respondents were aged 36 years and above (53.33%) followed by 26 to 35 years (38.89%), 19 to 25 years (6.67%) and 18 years and below (1.11%). This shows that older people were involved in livestock production than young people probably because they are more experienced than the young generation. However, youth participation in livestock production in this study is high (46.67%). According to the Annual Agricultural Survey 2017, youth (15 to 35 years) participation in agriculture in Botswana is 5.6% (Statistics Botswana, 2019).

According to Table 2, 67.78% respondents had junior secondary education followed by non-formal education (13.33%), primary education (5.56%), never attended school (5.56%), senior secondary education (4.44%), and tertiary education (3.33%). The literacy level in this study was 94.44% which indicates that the respondents were likely to consume extension messages and/or try to adopt new technologies with ease. Opoku-Amankwah and Brew-Hammond (2009) stated that people who can read and write are able to keep records in trade and agriculture.

Animals affected by stable flies

Stable flies and other flies such as horn flies, horse flies, house flies and bow flies are considered troublesome to goats (Talley, 2015). Table 3 shows that sheep and goats are affected by stable flies (47.78%) more than other animals followed by cattle (34.44%), dogs (3.33%) and calves (2.22%). In consonance with this finding, the North West district First Sub-Council Meeting of 2021/2022 stated that high mortalities due to stable flies were recorded in small stock compared to other livestock such as cattle, donkeys and equines (North West District Council Report, 2021). Data from the Sub-Council revealed that 2282 small stock, 310 cattle and 7 donkeys were killed by stable flies. Sheep and goats appear to be vulnerable to stable flies probably because of their short tails which make it difficult for them to keep away stable flies. Compared to large stock (cattle and equines), small stock has thin coat layers which enable the stable flies' sharp proboscis to penetrate easily during feeding activity. Although stable flies feed mainly on large animals such as horses, donkeys and cattle for a blood meal, in the absence of these animals they will bite goats, sheep, pigs, pets and humans (Kaufman and Weeks, 2019). According to ElAshmawy et al. (2021), cattle react to biting stable flies with an aggregating behavior known as bunching, which reduces grazing or feed consumption leading to a decrease in cattle productivity and welfare.

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Table 2 - Demographic characteristics of respondents in the study area

Category	Frequency	Percentage	Chi-square test p-value*
Marital status			
Single	35	38.89	
Married	49	54.44	
Divorced	5	5.56	
Other	1	1.11	
Total	90	100	0.0001
Sex			
Female	46	51.11	
Male	44	48.89	
Total	90	100	0.8330
Age			
≤ 18 years	1	1.11	
19-25 years	6	6.67	
26-35 years	35	38.89	
≥ 36 years	48	53.33	
Total	90	100	0.0001
Education			
Primary school	5	5.56	
Junior secondary school	61	67.78	
Senior school	4	4.44	
Tertiary	3	3.33	
Non-formal education	12	13.33	
Never attended school	5	5.56	
Total	90	100	0.0001

*The categories of the respective variables have significantly different frequencies if the chi-square test p-value less than 0.05.

Table 3 - Animal mostly affected by stable flies in the study area

Animal	Frequency	Percentage
Small stock	43	47.78
Dogs	3	3.33
Calves	2	2.22
Cattle	31	34.44
Chi-square p-value	0.0001*	

*Animal categories have significantly different frequencies since the chi-square test p-value is less than 0.05.

Table 4 - Part of the day in which stable flies attack animals

Time	Frequency	Percentage
Morning	28	31.11
Afternoon	6	6.67
Evening	53	58.89
Night	3	3.33
Total	90	100
Chi-square p-value		0.0001*

*Time categories have significantly different frequencies since the chi-square test p-value is less than 0.05.

Time of stable flies occur

The frequency analysis output showed that 53% of the respondents mentioned that stable fly outbreak occurs in autumn (March and April) while the remainder said it occurs in summer (November to February). These frequencies were not statistically different (P>0.05) though it is expected that precipitation in summer favours the outbreak of stable flies. Actual measurement of the prevalence of the stable flies in the study area might prove otherwise compared to the respondents' perception. Kaufman and Weeks (2019) observed that stable fly abundance is closely linked to rainfall. Previous study by Evert (2014) at Karan Beef Feedlot in South Africa showed that high rainfall contributes to the abundance of stable flies. Furthermore, Gillies et al. (2008) and Erasmus (2015) reported that environmental factors such as temperature, humidity and rainfall are positively correlated to stable fly outbreaks. In Botswana, November to February is the rainy season and during this period temperatures are high in the North West district and the rest of the country. These factors (i.e., temperature and moisture) favor the outbreak of stable flies. A related study by Pitzer et al. (2011) on equines in Florida State in the United States reported that stable flies can be active throughout the year but feeding activity is greatest between January and April (fall) depending on the amount of rain. This indicates that rainfall is an important factor in stable fly outbreaks.

Approximately 59% of the respondents mentioned that stable flies attack animals in the evenings followed by mornings (31.11%) (Table 4). These findings are consistent with Semelbauer et al. (2018) and ElAshmawy et al. (2021) who stated that temperature and relative humidity are the most important factors that influence feeding activity of stable fly. The authors observed stable flies' seasonal activity to be large at the end of summer with a second smaller peak before the end of the flight season. Kaufman and Weeks (2019) reported that in warm weather, stable flies feed on their hosts during the early morning and late afternoon while in cooler weather feeding is in the middle of the day. Berry and Campbell (1985) demonstrated that stable fly feeding activity is at peak at 24-30 ^{o}C. In another study, Lendzele et al.

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(2019) reported that the pest's daily activity peak was between 14 hours and 16 hours with a mean temperature of 31 °C, a mean wind speed of 1.5 m/s, and a mean humidity of 50%. Showler and Osbrink (2015) reported that stable flies bask in the sun on the sunny side of animals when the temperatures are low, indicating that temperature positively correlates to stable fly feeding activity. In Thailand, Masmeatathip et al. (2006) found that stable flies' feeding activity was high between 08:00-10:00 hours. In disagreement with the current findings, Thomas et al. (1989) in southern Nebraska in the United States observed that stable fly feeding activity in feedlot animals was higher at 1400 hours.

Control measures against stable flies

Although many researches in different parts of the world have been carried out on the ways of eliminating stable flies, none of these measures have been reported to be successful. From Table 5, 53.33% of the respondents used wood smoking as a control measure against stable flies followed by migration (relocation of livestock) to unaffected areas (35.55%) and dips (11.11%). The 2021/2022 North West District Council First Session reported that 26 cattle crushes in Sehithwa, Tsau, Shorobe and Bodibeng extension areas were affected by stable flies and that 198 farmers were assisted with acaricides to dip their animals (North West District Report, 2021). The respondents reported that they experienced high mortalities due to late provision of acaricides. This indicates that farmers in the study area were not taking it upon themselves to buy dips but wait for government, international organizations such as United Nations Development Programme and the private sector to supply them with dips. During the 2018 stable fly outbreak some company in Maun (the capital of North West district) donated acaricides to farmers around Sehithwa extension area. It is therefore important that the extension services encourage farmers to purchase acaricides and dip their animals in time to reduce losses due to stable flies.

Table 5 - Common practices used by farmers to control stable flies in the study area

Animal	Frequency	Percentage
Dips	10	11.11
Migration to unaffected areas	32	35.55
Wood smoking	48	53.33
Total	90	100
Chi-square p-value	0.0001*	

*Practice categories have significantly different frequencies since the chi-square test p-value is less than 0.05.

As mentioned earlier, wood smoking was the most common control measure used against stable flies in the study area. Green grass, old tyres and cow dung were burnt to produce smoke to render pests inactive. Wood smoking is cheaper compared to dips but is unfriendly to the environment. According to Hogsette et al. (1987), burning repeatedly effectively kills stable fly larvae and pupae. In this study, only 11.11% of the respondents used dips possibly because majority of them did not know how to mix and apply dips. About 82% of the respondents mentioned that they could not afford to purchase acaricides to use against stable flies as they were expensive. Failure by majority of respondents to use dips might point to inadequacy of technical support from the extension services (government or private). Foil and Hogsette (1994) stated that treatment of animals with residual insecticides can help in controlling stable flies with thorough application of the chemical to the lower body parts of livestock being important.

Seventy-five percent of the respondents mentioned that they could not relocate their animals from the areas affected with stable flies to unaffected areas due to high labour costs required for migration. Relocating animals from areas affected with *S. calcitrans* to unaffected areas is likely to result in the spread of this pest and diseases to areas that were not affected before and hence lead to more animal deaths. Rochon et al. (2021) reported that stable flies are associated with the mechanical transmission of several pathogens that cause diseases in animals. According to Kaufman and Weeks (2019), stable flies can transmit the pathogens that cause diseases such as anthrax, equine infectious anemia, and anaplasmosis. Furthermore, wounds resulting from bites can also become secondarily infected by opportunistic pathogens. Again, some animals are likely to get astray during the relocation while others might die from thirst and stress due to being trekked long distances. In addition, relocation of animals to new areas could lead to increased incidences of stock theft and overgrazing, as well as, mortalities due to adaptation challenges. Kneeland (2011) stated that integrated pest management is the most recommended pest control measure as it involves a combination of different pest control measures. Other control measures that can be effective in reducing stable fly populations include the use of modified traps, and using either treated targets or solar-powered electrocution grids (Foil and Hogsette, 1994).

Role of extension in the control of stable flies

About 97% of the respondents said that support from Ministry of Agricultural Development and Food Security was inadequate. Seventy-nine percent of the respondents reported that Department of Veterinary Services (DVS) provided acaricides on time (*i.e.*, before the rainy season) to enable farmers prepare for the outbreak, whereas the remainder said support came late. In addition, the respondents said that the acaricides supplied to farmers by DVS were not enough for

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their herds as one liter of acaricides provided was shared by 2 to 3 households. Previous study by Seleka (2005) in Botswana reported that livestock management and husbandry practices are poor among smallholder livestock farmers who dominate the livestock subsector in Botswana. Moreover, Cook (2020) observed that acaricides such as larvicides and animal spray provide a quick knockdown to adult flies, indicating that dips are more effective than wood smoking. Recently, Barros et al. (2019) evaluated the susceptibility of *S. calcitrans* populations to cypermethrin in the state of Mato Grosso do Sul in Brazil and found that all the populations were resistant to cypermethrin, with resistance factors among field populations ranging from 6.8 to 38.6. The authors concluded that the intensive use of insecticides leads to the development of pyrethroid resistance in stable fly populations.

In this study, 97.77% of the respondents mentioned that they were not trained to use acaricides while a smaller percentage of respondents acknowledged receiving training on the use of acaricides. The training which was on livestock management was held in Nxaraga Rural Training Centre and lasted for 5 days. The fact that about 98% of the respondents did not receive training on the use of acaricides indicates that technical support to farmers is inadequate. This also indicates that extension service could be inadequate.

Impact of stable flies on the rural economy

Stable flies are pests of economic importance as they continue to cause great losses in animal production. The 2021/2022 North West District Council first session estimated small stock, cattle and donkey mortalities due to *S. calcitrans* to be 2282, 310 and 7, respectively (North West District Report, 2021). As the Government of Botswana buys a unit of small stock for beneficiaries of Government support programmes such as Livestock Management and Infrastructure Development and Poverty Eradication at 1000 BWP (100 USD), small stock losses due to stable flies is estimated to be 2 282 000 BWP (228200 USD). However, this value is underestimated as most farmers could not recall losses incurred or report losses to DVS. The cost of medication for the diseased animals is not known as the number of diseased animals could not be estimated.

As illustrated in Figure 2, the outbreak of stable flies has deleterious effect on the livelihoods of households in the study area. About 87% of the respondents mentioned that stable fly outbreaks contributed to poverty followed by starvation (16.67%) and loss of income (13.33%). The loss of animals due to stable flies render households which depend majorly on livestock production for a living more vulnerable to economic hardships, especially in the era of COVID-19 pandemic. The climate and soils in the North West district support livestock production than crop production. The respondents mentioned that they were no longer able to sell their animals due to their poor body conditions resulting from stable flies' bites.

Fifty-five percent of respondents said they lost nearly all their animals due to stable flies. This indicates that stable flies cause economic losses and hardships to livestock farmers in the study area; hence the need for intervention. Taylor et al. (2012) estimated economic national losses due to stable flies for each animal industry sector in the United States to be 358 million USD, 226 million USD, 360 million USD, 1.268 million USD for cow calf herds, cattle on feed, dairy cattle and pastured cattle, respectively. The authors estimated the median annual losses in animal production to be 139 kg of milk for dairy cows, 6 kg of body weight for pre-weanling calves, 26 kg body weight for pastured calves and 9 kg of body weight for feeder cattle of body weight. These values show that stable flies detrimentally affect animal performance, people' livelihoods and the economy in its entirety.

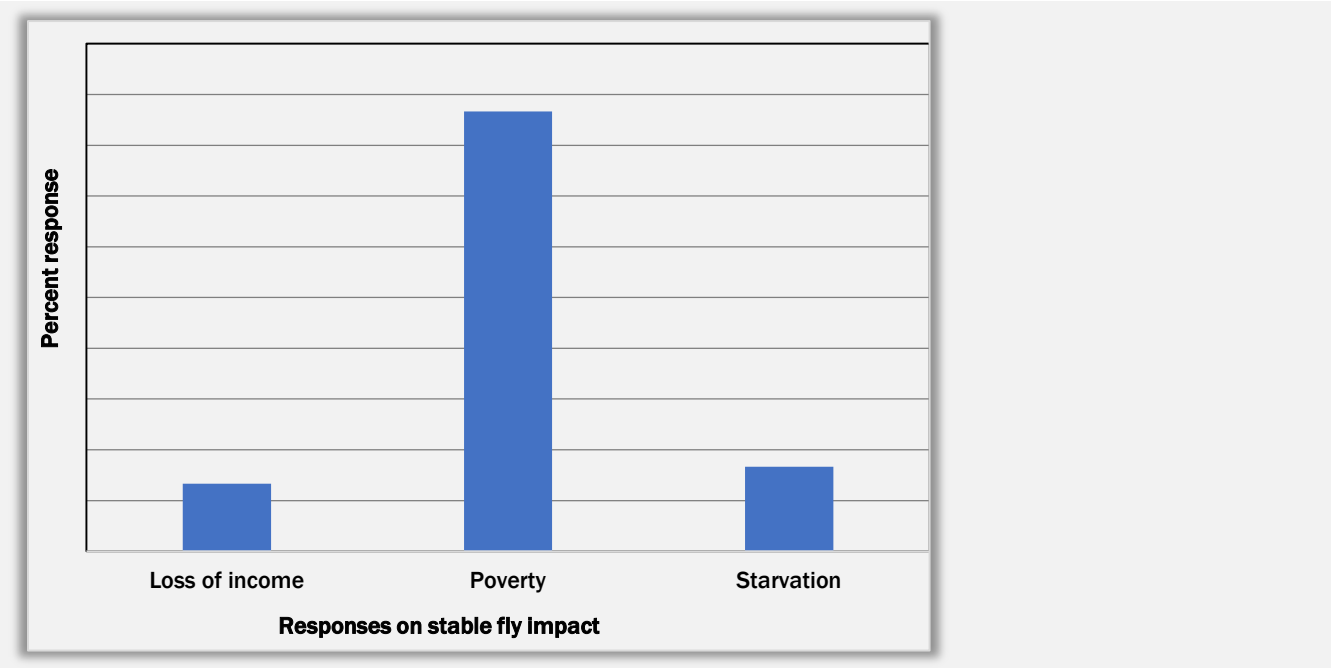

Figure 2 - Impact of stable flies on people's livelihood

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CONCLUSION

Stable flies cause economic losses in livestock production, thus affecting livelihoods of families in North West District and the rural economy in its entirety. Therefore, there is a need to come up with effective control measures against this pest. Although 53.33% of respondents said wood smoking was an effective control measure against stable flies, it is important that the respondents are encouraged to adopt other control measures such as integrated pest management to eliminate stable flies to avoid unnecessary losses.

Recommendations

1. Further research on stable fly ecology and biology in the North West district is required in order to come up with more effective control measures against this pest of economic importance.
2. Monitoring of stable fly for early detection of possible outbreaks using sticky traps must be carried out.

DECLARATIONS

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Authors' contributions

J.C. Moreki: Conceptualised the study, guided collection of data and wrote the manuscript.
K. Tjinyeka: Collected data and participated in the writing of the manuscript.
K. Tlotleng: Edited and critiqued the manuscript.
J. Makore: Assisted with statistical design and analysis and edited the manuscript.
M.I. Moseki: Edited the manuscript and provided some relevant literature.

Availability of data

Data can be made available to the journal upon request.

Conflict of interest

All the authors consented to the publication of this manuscript. The authors declare no conflict of interest.

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NEW METHODS FOR DETOXIFICATION OF HEAVY METALS AND MYCOTOXINS IN DAIRY COWS

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ABSTRACT: Among the many environmental and industrial factors that adversely affect the soil, the pollution with heavy metals and mycotoxins occupies a special place in livestock breeding. This study aimed to demonstrate methods of treating and pharmacological correction of a toxic state intensified by heavy metals and mycotoxins in cattle using native drug as mycotoxin-deactivating feed additive. A total of 20 highly productive Holstein cows aged 3-4 years were divided into 2 groups, based on clinical and biochemical parameters. The experimental group (n=10) fed a diet supplemented with 4% sorption complex and a drug based on *Silybum marianum* and ursodeoxycholic acid as a hepatoprotector named "Hepaton-vet". The control group (n=10) was injected with a complex of sorbents (consisting of perlite, vermiculite, and polyphepan in equal proportions) at a dose of 4% of the daily intake of food, and the rest of the treatment was carried out with the help of daily infusion therapy. So in the feed samples, only the T-2 and Deoxynivalenol (DON) indicators exceeded the normal value by 1.66% and, 3%, respectively. Thus, the practical efficiency concerning T-2, aflatoxin and Deoxynivalenol were to 100%, 86%, 18%, respectively. Cadmium in compound feed was lower by 44%, in comparison with the maximum permissible concentration, followed by 53.3% in hay and 78% in silage. The amount of lead in compound feed and silage was 78%, and it was 35% in the hay. In conclusion, the use of a complex of sorbents, together with newly developed component "Hepaton-vet", led to positive results, allowing for the identification of several effects that influenced the metabolic processes in the liver, which was confirmed by the results of morpho-biochemical blood tests and clinical diagnostics of the animals' condition.

Keywords: Cattle, Heavy metals, Mycotoxins, Silage, Sorption complex.

INTRODUCTION

The co-occurrence of organic pollutants and heavy metals in soil is of great concern to scientists around the world (Ye et al., 2017), but the versatility of this problem and the lack of knowledge about the relationship between soil ecology and the fate of pollutants limits the development of a specific control strategy (Yang et al., 2015). Despite this, various committees and funds have already been created around the world to monitor, assess the state, and forecast changes in the state of the environment, created to highlight the anthropogenic component of these changes against the background of natural processes (Vorotnikov et al., 2020).

Among the many techno-genic factors that adversely affect the soil cover, soil pollution with heavy metals (HMs), such as zinc, lead, and cadmium, occupies a special place. In addition, mycotoxins also cause great damage to the entire agro-industrial complex (Gallo et al., 2015) Mycotoxins can cause many adverse health effects and pose serious health risks to both humans and livestock (Haque et al., 2020). Thus, the Codex Alimentarius Commission (a joint intergovernmental body of FAO and WHO, based on the recommendations of JECFA, develops and adjusts international standards and codes of practice to limit the content of mycotoxins in certain foods (Cozma et al., 2017). The chemical composition of feed and food can be considered a reflection of the chemical pollution of the environment in general and the soil in particular (Rodrigues and Römkens, 2018). Control of toxic elements is necessary to block them in any part of this ecological chain before entering to agricultural cycles (Rigby and Smith, 2020).

The maximum level of concentration of a particularly toxic element in animal feeds and diets must be considered taking into account the duration of use of contaminated feed products, methods of their processing, and storage. Prolonged exposure to heavy carcinogenic elements in small amounts, imbalance of diets in essential nutrients, keeping animals in poor conditions can also have a devastating effect on the body (EFSA's Panel on Additives and Products or Substances used in Animal Feed, FEEDAP, 2016).

At the same time, it should be taken into account that agriculture is a rapidly developing industry, and by increasing the concentration of livestock, it is difficult to provide all the necessary parameters for raising animals. And by creating

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natural conditions of detention, it is impossible to ensure the pace of production. Thus, to obtain high results, it is necessary to strike a balance between the desired profit and the physiological capabilities of animals. Particularly in cattle rearing, mismanagement of feeding program and dietary contaminations with heavy metals and mycotoxin often leads to metabolic disorders of the liver (Kan and Meijer, 2007; Semenenkoet al., 2017). Degenerative obesity of the liver, leading to necrosis of hepatocytes and their disintegration with subsequent autolysis, can occur with chronic intoxication with spoiled silage with a high content of butyric acid, vegetable and mineral poisons such as alkaloids, lupine, phosphorus, arsenic, mercury, as well as feeding animals with residues technical feed processing (Dhama et al., 2015).

In the case of oral poisoning, the absorption of heavy metals occurs in an ionized form in the duodenum and the initial section of the jejunum (Foulkes, 2000). In the blood, metals circulate in the form of ions in combination with amino acids, forming a strong bond with blood proteins, therefore, within several months, metals are distributed and deposited in all organs (Elder et al., 2015; Oymak et al., 2017). Especially in large quantities, metals and arsenic accumulate in the kidney and liver tissues, because. They have an increased content of metallothionein protein, rich in thiol groups (Blanco-Penedo et al., 2006). The excretion of metals occurs through the kidneys, liver, sweat, and salivary glands, which is accompanied by damage to these organs and tissues (Lehman-McKeeman, 2008).

There is a natural relationship between the toxicity of a metal and its physicochemical properties (Jaishankar et al., 2014), such as: the higher the atomic weight of the metal, the more toxic its preparations; the stronger the association with proteins, the higher the toxicity; organic metal compounds are more toxic than inorganic ones; as the valency of a metal decreases, its toxicity increases; with an increase in the degree of dissociation of the metal salt, toxicity increases.

When animals fed by diets contaminated with toxicogenic fungi, mycotoxins can be detected in meat and dairy products (Yang et al., 2020). The mechanism of their action is due to the blocking of vital amino acids - alanine, tyrosine, tryptophan and the formation of amines, which, even in small quantities, can adversely affect blood vessels.In acute poisoning, for example, with aflatoxin, the target organ is the liver, in which necrosis and fatty degeneration develop, and in chronic poisoning, cirrhosis, and primary cancer (Burcham, 2014).

Detoxification of mycotoxins and heavy metal in feed is a new challenge of feed producing companies (Elliott et al., 2020; Neculai–Valeanu et al., 2020). Thus, present study offers a treatment regimen with native drugs (plant-based medicines) that will reduce overall intoxication and obtain high-quality environmentally friendly products. The purpose of this study is to demonstrate new ways of treating and pharmacological correction of a toxic state intensified by heavy metals and mycotoxins in cattle using native drugs.

MATERIALS AND METHODS

In present experiment, the content of mycotoxins was determined by enzyme-linked immunosorbent assay (ELISA) on Agra Quant kits (Agra Quant® Deoxynivalenol ELISA test, Australia).To quantitatively characterize the sorption capacity of the sorption complex (perlite, vermiculite, and polyphepan), the indicator "Practical efficiency factor - PKPD" was adopted. The sorbent capacity is determined as a percentage, by the difference between adsorption and desorption. Sorption of mycotoxins was determined quantitatively at different pH, simulating the change in the acidity of the environment in the digestive tract of animals. The percentage of adsorption and desorption was measured during the in vitro test. Heavy metals in feed and milk samples were determined by the atomic absorption spectrometry according to Smirnov et al. (2008).

Production experiments were carried out at the Agricultural Production Cooperative (APC) "Kolkhoz Leninsky Put", Vasilievskoye village, Pushkinogorsk district, Pskov region, on highly productive Holstein cows aged 3-4 years. The cows participating in the experiment were housed. Feeding was carried out 2 times a day using a typical diet: corn silage, alfalfa haylage, bagasse, alfalfa hay, molasses, and compound feed. Access to water is free, *ad libitum*.

The animals were divided into 2 groups. The diagnosis was made based on clinical and biochemical parameters. The first group (n=10) was administered a sorption complex with food at a dose of 4% of the daily norm, and a drug based on *Silybummarianum* and ursodeoxycholic acid - "Hepaton-vet" (experimental prototype in the form of an emulsion, developed by the Department of Pharmacology and Toxicology of the Federal State Budgetary Educational Institution of Higher Education "SPbGUVM") was used as a hepatoprotector, which described by Kalyuzhny et al. (2021). The second group (n=10) also a tea complex of sorbents at a dose of 4% of the daily intake of food, and the rest of the treatment was carried out with the help of daily infusion therapy.

The criteria for the therapeutic efficacy of the sorption complex (based on perlite, vermiculite, and polyphepan, in equal proportions) and the drug "Hepaton-vet" were positive changes in the clinical condition of cows, the dynamics of blood homeostasis (Stepanov et al., 2021). The assessment of the clinical condition of the experimental animals was carried out daily, biochemical studies of blood serum - in the middle of the experiment and at the end of the experiment, morphological studies of blood - at the end of the course of drug therapy.

Statistical analysis

Statistical processing of the obtained data was carried out using Microsoft Excel and Statistica 6.0 programs using Student's t-test. The results are considered statistically significant at $p<0.05$.

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Ethical regulation

The procedure of the experiment fully complied with the Directive of the European Parliament and the Council of the European Union 2010/63/EU of September 22, 2010 on the protection of animals used for scientific purposes, and was also approved by the Bioethics Committee of the Federal State Budgetary Educational Institution of Higher Education "Saint-Petersburg State University of Veterinary Medicine".

RESULTS AND DISCUSSION

The toxicological evaluations of the feed showed that both feed and silage on the farm contain mycotoxins, which is reflected in Table 1. Table 1 shows an assessment of the content of mycotoxins in feed in the agro-industrial complex "Kolkhoz Leninsky Put", for the complex selection of sorbents in phytobiotics, when analyzing the quantitative characteristics of the sorption capacity (indicator of the practical efficiency of the sorbent in percent). Thus, the content of T-2 toxin in the silage was 25%, higher than those in compound feed and silage, zearalenone was 10% higher, also. The content of aflatoxin exceeded was 60% higher than the than in compound feed and silage. So in the feed samples, only the T-2 indicator exceeded the normal value by 1.66%, Deoxynivalenol (DON) by 3%. To quantitatively characterize the sorption capacity, the Practical Efficiency Factor (PEF) of the sorbent was determined as a percentage, by the difference between adsorption and desorption. The data is shown in Figure 1. Figure 2 shows the content of cadmium, lead and mercury in different types of feed of the APC "Kolkhoz Leninsky Put".

Table 1 - Evaluation of the content of mycotoxins in feed in the agricultural cooperative "Kolkhoz Leninsky Put"

Content in feed, mg/kg / Mycotoxins	Compound (concentrate) feed; KK-60-1-3	Hay	Silage
T-2 mycotoxin	0.061±0.01	0.071±0.01	0.075±0.012
Zearalenone	0.067±0.01	0.082±0.008	0.11±0.03
Aflatoxin	0.0038±0.005	0.066±0.015	0.064±0.017
Deoxynivalenol (DON)	1.03±0.2	2.52±0.4	1.0±0.3
Ochratoxin	0.0001±0.00015	0.0032±0.0016	0.004±0.001

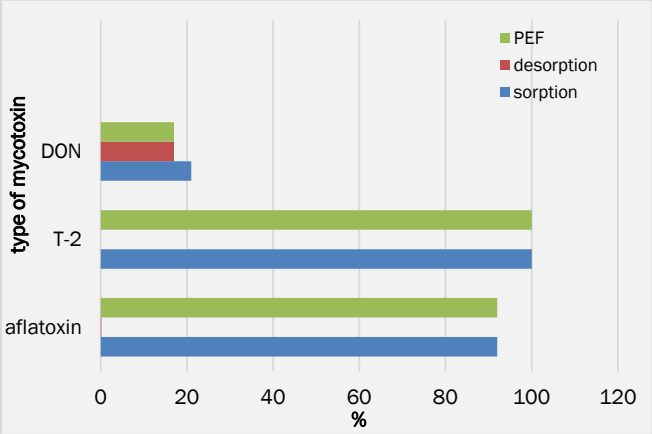

Figure 1- Sorption capacity of the sorption complex, % (DON= Deoxynivalenol; PEF= Practical Efficiency Factor),

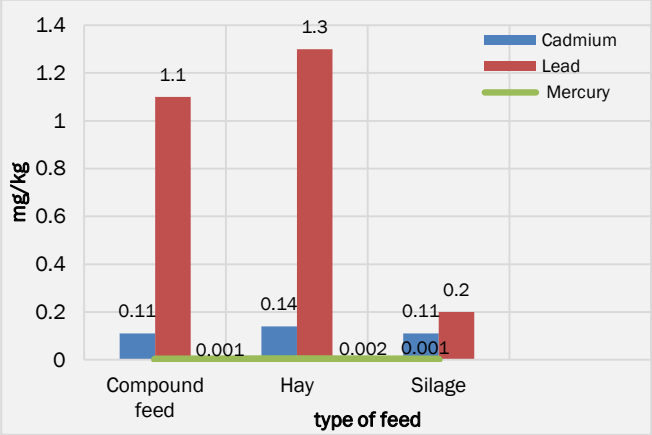

Figure 2 - The ratio of heavy metals in feed of different types at the Agricultural Production Cooperative (APC) in Pskov region (measures presented in %)

Thus, the studied feeds also turned out to be safe concerning heavy metals. Thus, cadmium in feed, in comparison with the maximum permissible concentration (MPC), was lower by 44%, in the hay by 53.3%, in silage by 78%. The largest amount of cadmium was found in hay 0.14 mg/kg, 27% more than in compound feed and silage. The amount of lead in compound feed was below the limit values by 78%, in the hay by 35%, in silage by 78%. The content of mercury, as well as in the soil, was the lowest and amounted to 0.001 mg/kg in mixed fodder and silage, 0.002 mg/kg in the hay.

The state of intoxication and pathology of the liver was diagnosed based on anamnesis, clinical examination, palpation, percussion, auscultation, and the results of morphological and biochemical blood tests. Clinical examination of cows with signs of hepatosis was characterized by symptoms, manifested by general depression, muscle weakness, progressive emaciation against the background of a decrease in appetite. On the part of the gastrointestinal tract, disorders of the proventriculus (hypotension and atony), stagnation of food masses in the book, slowing down of intestinal peristalsis, as well as diarrhea, followed by constipation, were noted. The body temperature was normal or decreased to the minimum limits of the norm.

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In some cases, in animals on the mucous membranes of the eyes and eyelids, anemia, icterus, as well as hemorrhages of various scales, from pinpoint to diffuse, were recorded. Peeling of the epidermis was observed on the surface of the skin. With jerky palpation of the abdominal wall, the liver area was painful, and in most cases, there was a displacement of the percussion border of the liver. The boundaries of the area of hepatic blunting were localized in the range from the 13th to the 9th rib, while the shape of the percussion border was palpable in the form of a half-lobe or an elongated rhombus. To clarify the diagnosis, blood was taken from the animals for laboratory tests. A general blood test of cows found that the most significant differences between sick cows (with toxicity) and clinically healthy animals were revealed in the level of erythrocytes and hemoglobin, the values of which were lower than in healthy cows by 21.3% and 25.2%, respectively.

It was found that during the experimental period in the experimental groups, the hemoglobin content increased by 3.9% and 3.6%. According to the level of erythrocytes, there was a tendency to increase their number by 12.1% (experimental group 1) and 10.2% (experimental group 2). Color index values increased by 10.6% and 11.3%, respectively. Such a picture may be the result of regenerative processes in the hematopoietic system and positive changes in the work of the blood depositing organ - the liver. Whereas in animals that did not receive the drug, the number of red blood cells decreased by 14.2% compared with the background values of the group (Table 2).

Under the influence of drugs (complex of sorbents and "Hepaton-vet") in cows of both experimental groups, there was a decrease in the level of leukocytes by 23.2% and 21.4% relative to the background (Table 2). In control animals, on the contrary, signs of leukocytosis were noted in the blood.In cows with clinical signs of general intoxication, deviations were observed in several biochemical parameters of blood serum, manifested by hypoproteinemia and hypoalbuminemia against the background of a decrease in the protein-synthesizing function of the liver (the concentration of total protein and albumin fraction was 74% and 82% of the corresponding indicators of healthy cows). The level of urea was reduced in 61% of the samples, low glucose content was noted (Table 3).

Table 2 -Hematological indices of cows after the application of a complex of sorbents and the drug "Hepaton-vet" (M±m; n=10)

Indicator	Experimental group	Control group	P Value
Leukocytes (10^9/l)	7.4±0.17[b]	10.5±0.26[a]	P ≤ 0.05
Eosinophils (10^9/l)	0.4±0.02	0.5±0.02	-
Neutrophils (10^9/l)	2.1±0.09	1.9±0.19	-
Lymphocytes (10^9/l)	4.6±0.23	7.6±0.18	-
Monocytes (10^9/l)	0.3±0.03	0.3±0.02	-
Eosinophils (%)	4.0±0.07[b]	4.5±0.06[a]	P ≤ 0.05
Stab neutrophils (%)	2.2±0.16	2.4±0.18	-
Segmented neutrophils (%)	24.3±1.93	15.9±1.77	-
Lymphocytes, %	64.9±4.28	74.3±4.62	-
Monocytes, %	3.7±0.29[a]	2.9±0.32[b]	P ≤ 0.05
Erythrocytes (10^{12}/l)	7.44±0.38	5.21±0.4	-
Hemoglobin (g/l)	109.4±5.19[a]	93.6±4.35[b]	P ≤ 0.05
Hematocrit (%)	28.4±1.46	21.4±2.13	-
Average erythrocyte volume (fL)	49.7±2.5	43.5±1.6	-
Average content of hemoglobin in an erythrocyte (pg)	17.4±0.67	17.4±0.43	-
Color indicator (units)	0.96±0.06	0.83±0.04	-
Platelets (10^9/l)	316.4±26.2	232.8±34.6	-
Average platelet volume (fL)	7.0±0.51	5.1±0.34	-
Thrombocrit (%)	0.5±0.04	0.3±0.03	-
erythrocyte sedimentation rate (ESR)	1.2 ±0.06	1.4±0.03	-

Control group: ate a complex of sorbents (consisting of perlite, vermiculite, and polyphepanin equal proportions) at a dose of 4% of the daily intake of food; Experimental group: fed a diet supplemented with 4% sorption complex and a drug based on *Silybum marianum* and ursodeoxycholic acid as a hepatoprotector named Hepaton-vet.

Table 3 - Serum biochemical parameters of cows with hepatosis when using the "Hepaton-vet" (M±m; n=10)

Indicator	Experimental group		Control group	
	10th day	21st day	10th day	21st day
Glucose.mM/l	2.09±0.12	2.76±0.21	2.32±0.14	1.84±0.11
Cholesterol.mM/l	3.94±0.3	5.82±0.6*	3.2±0.3	3.6±0.2
Triglycerides.mM/l	0.15±0.04	0.18±0.03	0.16±0.04	0.13±0.04
Total bilirubin. μM/l	11.8±0.6	6.13±0.3**	13.1±0.7	11.2±0.5
Ca.mM/l	2.22±0.06	2.61±0.07	2.11±0.11	2.18±0.13
P.mM/l	1.4±0.1	2.4±0.5	1.4±0.1	1.7±0.2
Carotene. mg %	0.43±0.03	0.61±0.02	0.26±0.02	0.22±0.01

* p ≤ 0.05; **p ≤ 0.01

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Table 4 - Content of heavy metals in milk of cows for 21 days. (M±m. n=10)

Indicator	Experimental group		Control group	
	10th day	21st day	10th day	21st day
Cadmium (Cd)mg/l	0.006±0.001	0.003±0.0013	0.004± 0.0016	0.0046±0.0012
Lead (Pb)mg/l	0.034±0.004	0.0113±0.005	0.0175± 0.005	0.0206±0.003
Mercury (Hg) mg/l	-	-	-	-

* $p \leq 0.05$; **$p \leq 0.01$

Against the background of increased destructive processes in liver cells, lipid peroxidation is activated, leading to the destruction of hepatocyte membranes, which causes the release of transamination enzymes - transaminases into the bloodstream. At the same time, there is a more pronounced increase in aspartate aminotransferase, on average, 1.61 times compared with the same indicator in the blood of healthy cows, which is a diagnostic criterion for the chronic course of the pathological process in liver cells, since Aspartate transaminase (AST) belongs to mitochondrial enzymes, the concentration of which increases sharply with destruction and necrosis of hepatocytes. The decrease in alanine aminotransferase was less pronounced - by 21.3-23.9% of the species norm.

An increase in the activity of alkaline phosphatase (by 2.02 times) may indicate the presence of cholestasis accompanying hepatosis provoked by toxins. The activation of this process can also explain the increase in the level of bilirubin in the blood (by 2.1-3.6 times) as a result of damage and compression of the bile capillaries. In all samples, a decrease in the level of triglycerides was recorded (Table 3), which indicates hypolipoproteinemia observed in parenchymal liver diseases. The values of carotene were low (the decrease was 1.57-4.12 times from the average limits of the species norm), which could be caused by general metabolic disorders in the body of cows, as well as deterioration in the functioning of the carotene-depositing system of the liver (Kalugniy et al, 2021).

Thus, under the influence of complex treatment with the use of sorbents and the drug "Hepaton-vet",significant changes occurred in the biochemical homeostasis of cows. So, at the beginning of the experimental period, relative hypoproteinemia was observed in both experimental groups. At the same time, the type of proteinograms was characterized by a significant decrease in the content of albumin due to deep dystrophic changes in hepatocytes, leading to disruption in the biosynthesis of proteins of this fraction with a tendency to increase in γ-globulins (as a result of compensatory redistribution of the protein spectrum of blood serum). But already by the 12th day of therapy, there was a gradual normalization of the fractional composition of blood serum, due to an increase in the level of albumin against the background of a decrease in the level of gamma globulin fraction. At the same time, the concentration of albumins in the first group, with the use of "Hepaton-vet", by the end of the experiment increased relative to the indicators of control animals by 21.2% and 17.3%, respectively, and the concentration of the gamma globulin fraction decreased by 37.3% and 40%.

An important factor in the study of sorption medicines is their ability to absorb the main toxicants, in particular heavy metals. Heavy metals in milk samples were determined by atomic absorption spectrometry. Samples were taken from animals of the first and second groups.

At the beginning of the study, the presence of heavy metals in the experimental and control groups was significantly below the maximum allowable values. Mercury was not detected in any of the samples. The amount of cadmium in the control group by the end of the experiment increased by 15%, lead by 17%. In the experimental group, where the sorption complex and "Hepaton-vet" were used, we observed the reverse dynamics, the amount of cadmium decreased by 50%, lead by 66%. However, it should be noted that the level of heavy metals in milk in both the experimental and control groups by the end of the experiment remained within the acceptable range.

It was established that therapy with an integrated approach aimed at reducing general intoxication and maintaining the liver function, using a sorption complex based on perlite, vermiculite, and polyphepan, and the drug "Hepaton-vet", had a positive effect on the clinical and metabolic status of cows. Clinically, this was manifested by an improvement in appetite, activation of digestion processes, and normalization of the motor function of the scar. The number of ruminant periods increased on average up to 5 times per day. Starting from the third week of therapy, the condition of the skin and mucous membranes of the body improved in cows. Against this background, in animals of the control group, the clinical picture of fatty degeneration of the liver persisted.

Phytobiotics (phytosorption) are natural medicines or feed additives of plant origin, and there is numerous published studies related to potential of these components for detoxification of mycotoxins and heavy metals in farm animals (Holandaand Kim, 2020; Holanda et al., 2021a, 2021b). Essential oils that are part of phytobiotic complexes, unlike antibiotics, have a selective antimicrobial effect, inhibiting the growth of pathogenic microflora in the gastrointestinal tract, and at the same time creating conditions for the growth of "useful" probiotic microflora (Abd El-Hack et al., 2021; Krivonogova et al., 2021). The development of green technologies that make it possible to economically and effectively clean the gastrointestinal tract of animals, along with the expansion of the range of sorption preparations, is currently

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promising. One of such technologies can be the addition of phytosorption complexes (FSK) to the feed (Awad et al., 2010; Nadziakiewicza et al., 2019). Known effective sorption preparations based on minerals and mineral phytosorbents are given in the following articles (Yakovlevaet al., 2016; Dauletet al., 2019). However, phytosorption complexes are more promising due to the clinical efficacy comparable to sorbents of another origin (Tiwari et al., 2006; Torok et al., 2017), while they practically do not affect livestock products, are safer in pharmaco-toxicological respect, their registration is greatly simplified by the bio waiver procedure. The joint use of phytosorption complexes together with hepatoprotectors is, in many respects, an innovative idea (Ponamarevand Popova, 2020), but is one of the most effective methods of pharmacological correction of such pathologies, taking into account their pathogenesis (Patent No. 2742414 C1).

CONCLUSION

In conclusion, based on findings of present study, it is possible to evaluate the targeted treatment of animals, and we can no longer talk about the group, but the individual treatment of productive animals based on the geographical location of the enterprise, and, consequently, cost reduction and, as a result, obtaining high-quality and safe products. Thus, the study of feed for mycotoxins showed a high degree of contamination. And although the data of the study of mixed feed show the level of mycotoxins, on the verge of acceptable values, nevertheless, such feed can subsequently harm the body. The level of productivity was lower by 11.42% in cows of the control group, which received a normal diet. During the 21 days of the experiment, dietary treatment of Sorption complex - 60 grams per animal with food, "Hepaton-vet" - orally at a dose of 100 ml 1 time per day was efficient for detoxification of mycotoxin and heavy metals in cows. The proposed method, the use of a complex of sorbents, together with "Hepaton-vet", led to positive results, identifying several effects that influenced the metabolic processes in the liver, which was confirmed by the results of morpho-biochemical blood tests and clinical diagnostics of the animals' condition. Therefore, the use of complex sorption materials and hepatoprotectors, which allow leveling the negative impact of environmental factors, is very promising. The use of sorption complexes with newly developed component “Hepaton-vet”in veterinary medicine, as part of the transition to a highly productive and environmentally friendly agricultural economy, will make it possible to carry out a pharmacological correction of the toxic state intensified by heavy metals and mycotoxins in cattle using native preparations, as well as to create pure products.

DECLARATIONS

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EFFECT OF SUPPLEMENTATION OF PHYTO-ANTIOXIDANT (BAOBAB FRUIT PULP MEAL) ON REPRODUCTIVE PERFORMANCE OF RABBIT DOES DURING HEAT STRESS

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Supporting Information

RESEARCH ARTICLE

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ABSTRACT: A study was conducted to evaluate the influence of a phyto-antioxidant (baobab fruit pulp meal; BFPM) on the reproductive performance of heat-stressed rabbits does. A total of 50 New Zealand White crosses adult rabbit does of 12 months old were used in the present study. The rabbits were randomly allotted into five experimental treatment groups, with ten rabbits per treatment in a completely randomized design. The rabbits were fed diets supplemented with graded levels (0.0%, 2.5%, 3.5%, 4.5% and 5.5%) of BFPM. Parameters monitored were thyroxin and progesterone secretion, serum metabolites, reproductive traits, and physiological performance of the kitten. Initial thyroxin, progesterone, and serum metabolite levels were low in all the treatment groups, and significantly increased during and after pregnancy. BFPM significantly improved litter size and weight of litter and reduced rectal temperature of the kitten. Gestation period, kit weight at weaning, weight gain, and heart rates of kitten did not show any significant difference. It was concluded that reproductive performance of does during heat stress is enhanced by the supplementation of phyto-antioxidants and performance was higher in 5.5% inclusion level of BFPM.

Keywords: Antioxidant, Gestation, Heat stress, Rabbit, Reproductive hormones.

INTRODUCTION

The recent trend in the changes of food preferences of many citizens as a way of a healthy life style is increasing the demand for Rabbit meat (Petrescu and Petrescu-Mag, 2018). In Nigeria, farmers are constraint to meeting with this demand because of reproduction inefficiency of the rabbits as a result of heat stress (Mailafia et al., 2010; Oladimeji et al., 2021). Rabbits are vulnerable to heat stress because of their poor functional sweat gland. Hence rabbits can only tolerate narrow temperature range for effective reproduction (Marai et al., 2001). The expression of the growth and reproductive potentials is only possible when animals develop and establish a homeostatic equilibrium with their external environment (Marai et al., 2001).

Previous studies observed that high temperature and heat stress were significantly detrimental to rabbits and their reproduction efficiency. It has been shown that heat stress alters TSH secretion and triiodothyronine (T_3) and thyroxin (T_4) concentrations in serum, in rabbits (Marai et al., 2004), poultry (He et al., 2018; Vandana et al., 2020), and sheep (Todini, 2007). Heat stress has been implicated in promoting oxidative stress either through excessive production of reactive oxygen species (ROS) or decreased antioxidant defenses, including vitamin C (Chauhan, et al., 2014). Thyroid hormone (free T_3 and free T_4) levels were decreased in heat-stressed goats in an attempt to reduce metabolic rate and heat production (Sivakumar et al., 2010). Heat stress also reduces the level of progesterone and causes a loss of LH surge in sheep (Sejian, 2014). It has been reported that exposure to hyperthermia during pregnancy caused marked growth retardation of the adrenal cortex and a decreased population of somatotropin in the adenohypophysis in the off-springs (Ross et al., 1985). Exposing females to heat stress after fertilization caused decreases in the quality and quantity of embryos in cows (Hussin and Al-Taay, 2009; Luceño et al., 2020) and mice (Kahl et al., 2015) after superovulation, and caused decreases in fetal growth in pigs (Omidi, 2015), and beef cows (Kumar, et al., 2015). It has been demonstrated that heat stress induces oxidative stress and vitamin C as an important water-soluble antioxidant might reduce the adverse effects of heat stress (Oloruntola, et al., 2015). Based on my knowledge, the vitamin C and heat stress impact on thyroid hormone levels in rabbits has not been clarified.

The use of plants in different forms as alternatives to synthetic products which are relatively expensive and hazardous (because of their residual effect) as antioxidants and in alleviating heat stress is becoming more popular in the tropics (Dhama, et al., 2015; Valenzuela-Grijalva, 2017; Ogunwole and Mosuro, 2020). Plants and their parts could serve

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as phytobiotics and antioxidants to the livestock (Dhama et al., 2015; Valenzuela-Grijalva, 2017). Some phytochemicals in plants improve antioxidant, anti-microbial, feed flavour, and palatability which could result in increased feed intake and performance in animals (Valenzuela-Grijalva, 2017). These tropical plants are available because of their rapid growth which is enhanced by the prevailing and environmental factors (Valenzuela-Grijalva, 2017). Baobab has been described by (Sidibe and Williams, 2002; Bosch et al., 2004). The fruit pulp was reported to contain a high amount of vitamin C (Sena, 1998; PhytoTrade-Africa, 2009), it was reported to increase feed intake, weight gain and was effective in alleviating heat stress in rabbits (Anoh, 2017).

This study was designed to evaluate the effects of baobab fruit pulp meal supplement (as a phyto-antioxidant) on the reproductive performance of heat-stressed rabbit does.

MATERIALS AND METHODS

Ethical regulation

The procedures for care and handling of animals in this study were strictly followed in accordance with the code of ethics for animal experiment as stated in htpp://ec.europa.eu/environment/chemicals/lab_animal/legislation_en.htm.

Experimental site

This study was carried out at the Rabbit unit of the National Animal Production Research Institute (NAPRI), Shika-Zaria, Nigeria in March – Aprial 2015. Shika-Zaria lies between 11° 12' 42" N and 7° 33' 14" E at an altitude of 691 m above sea level. Zaria is about 245 KM from Abuja Nigeria's capital. The area is situated in the Northern Guinea Savannah Zone of Nigeria having an average annual rainfall of 1100 mm, which starts from May to September, and average ambient temperature and relative humidity of 17 °C – 25 °C and 20 – 41% respectively during the cold period (Mornings and August – Feb 2015) and 20 °C – 39 °C and 25% - 60% during the hot periods (afternoons of March – July 2015).

Housing

The animals were housed in perforated metallic hutches measuring 75 × 75 × 75 cm and raised 80 cm from the floor level. The hutches were thoroughly washed and disinfected with a locally made disinfectant and allowed to dry for one week before the animals were brought. Feed and watering troughs which were made of bunt clay were provided in each hutch. The rabbits were placed individually in clearly labeled cells.

Meteorological data of rabbit microclimate

The microclimate (ambient temperature and relative humidity values) within the rabbit house were recorded twice daily at 08:00 h and 15.00 h during the study period using a digital thermometer (Cocet®, Shenzhen-Guangdong, China). The data collected was used to compute the temperature humidity index (THI), an indicator of the thermal comfort level of the rabbits. The THI was calculated using the modified formula for the rabbit by Marai et al. (2001) as follows: THI = t - [(0.31 – 0.31 x RH) (t – 14.4)]

Where RH = relative humidity /100; and t = ambient temperature.

The values of THI obtained were compared to that classified for tropical regions as shown below:

1) < 27.8 = absence of heat stress; 2) 27.8 - 28.9 = moderate heat stress; 3) 28.9 – 30 = severe heat stress; and 4) above 30 = very severe heat stress.

Experimental animals, diet and design

A total of 50 adult rabbit does (New Zealand White crosses) of 12 months old were used in this study. The rabbits were randomly allotted into the experimental treatments of five treatment groups and five replicates. They were ten rabbits per treatment and each rabbit in a treatment served as replicate in a completely randomized design. Rabbits in group 1 were not supplemented and served as the control 0%; while those in groups 2–5 were fed diets, containing graded levels of BFPM, 2.5%, 3.5%, 4.5% and 5.5%, respectively. The baobab fruit pulp meal which was in powdered form was purchased from a local market in Zaria-Nigeria. The basal diet composition (kg) was as follows: Maize 35, Groundnut haulm 20, groundnut cake 10, soybean meal 10, rice bran 15, Bone meal 9.2, common salt 0.35, mineral-vitamin premix 0.25, dl-methionine 0.1 and lysine 0.1. The basal diet was formulated to meet the nutrient requirements of growing rabbits according to the recommendations of NRC-1995. The nutrient composition of the basal diet is shown in Table 1. Feed and water were served *ad libitum*. All recommended managerial practices were duly observed.

Table 1 - Nutrient composition of the experimental diets.

	Control	2.5%	3.5%	4.5%	5.5%
Metabolizable Energy (kcal/kg)	2200	2200	2200	2200	2200
Crude Protein (%)	16	16	16	16	16
Crude fiber (%)	10	10	10	10	10
BFPM Supplementation	0.00g	250g	350g	450g	550g

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Hormonal assay and serum metabolite evaluation

Blood samples (5 ml) were collected at 10 h through the ear vein of 5 rabbits in the treatment groups before mating, during pregnancy, and after kindling, respectively into a bottle without anticoagulant and allowed to clot. The clotted blood samples were centrifuged at 3000 rounds/minute for 15 minutes. The serum harvested was stored at -10^{o}C until when analyzed. Serum thyroxine and progesterone concentrations were determined for does with the use of commercially-available ELISA Kits (Diagnostic Procedure Corp®, Los Angeles, CA, USA) according to the manufacturer's instructions. Serum glucose, total protein, albumin, and cholesterol concentrations were evaluated using an auto-analyzer and Chemical Commercial Kits from Stanbio Laboratory Inc®. San Antonio, Texas, USA.

Reproductive performance of rabbit does

The rabbits were allowed to adjust to the treatment for four weeks before mating. Does were brought individually to be serviced by the buck (1 buck: 1doe / treatment). 7th day weight increment and abdominal palpation was used to confirm pregnancy. Does that were not pregnant were re-mated. Parameters monitored included, date of kindle, litter size, weight of litter, weight of kitten, survivability (%) of kitten at weaning and kitten weight at weaning. Data were obtained from 2 parities and the experiment lasted for 20 weeks.

Physiological performance of kittens

When the kittens were 3 weeks old, their rectal temperature (RT) and heart rate (HR) were measured. The readings were taken at 14.00 h to 15.00 twice a week for 3 weeks. Rectal temperature was measured with a digital thermometer, and HR was measured by counting the heartbeat of each rabbit representing their treatment for one minute with the help of a stethoscope. Weekly weight gain was also determined.

Statistical analysis

Data obtained from all the experiments were subjected to analysis of variance, using the General Liner Model Procedure of (SAS version 2.8, 2002). Significant differences among treatment means were separated using the pair wise difference (Pdiff) in the SAS package. Values of $P < 0.05$ were considered significant

RESULTS

The monthly Temperature Humidity Index (THI) inside the rabbitry during the experimental period is shown in Figure 1. THI in the mornings averaged 26.44^{o}C while the Afternoon THI averaged 28.74^{o}C. The THI values kept increasing from the month of February with a peak in May. There was a decline in THI in June. The values indicated that the month of February had absence of heat stress in the rabbit house.

Initial thyroxine was low across the treatments (Figure 2) and significantly increased during pregnancy. Throxine levels increased (60.9 (ng/ml), 69.87(ng/ml), 76.88(ng/ml) and 80.1(ng/ml) for 2.5%, 3.5%, 4.5% and 5.5% respectively) as the levels of BFPM increases in the diets with 4.5% and 5.5% recording the highest values. After kindling, thyroxine concentration decreased. Thyroxine concentration after pregnancy did not show any significant difference among the treatment groups. Progesterone concentration (Figure 3) were generally low before pregnancy and significantly ($P<0.05$) increased during and after pregnancy in BFPM treated rabbits. Rabbits in the BFPM treatments recorded significantly higher progesterone during pregnancy (21.67 ng/mL, 20.12, ng/mL 19.68 ng/mL and 19.25 ng/mL) compared to the control (18.04 ng/mL). Progesterone decreased as the levels of supplementation of BFPM in the diets increases. After kindling, it was observed that the control recorded a higher progesterone compared to the BFPM treated does.

During pregnancy, BFPM treatments recorded a significantly ($P<0.05$) high glucose, calcium and phosphorous compared to the control. Total protein was low in 2.5% group and phosphorous was low in 3.5% group. After kindling, total protein, albumin, triglyceride and phosphorous showed significant ($P<0.05$) difference among the treatment groups with the control having a significantly higher values than the BFPM treated groups except in triglyceride which was low. In the treatments with BFPM, treatments 3.4% and 4.5% performed better than treatments 2.5% and 5.5%

Litter size and weight of litter were significantly ($P<0.05$) increased in 3.5% and 4.5% BFPM inclusions (Table 3). Weight of kitten increased in 2.5% and 4.5%, while litter size at weaning was larger in 4.5 and 5.5% BFPM. Gestation period and weight of kitten at weaning did not show any significant difference.

The BFPM significantly ($P<0.05$) reduced rectal temperature; rectal temperature decreases as the inclusion of BFPM in the diet increases. Heart rate and weight gain did not show any difference.

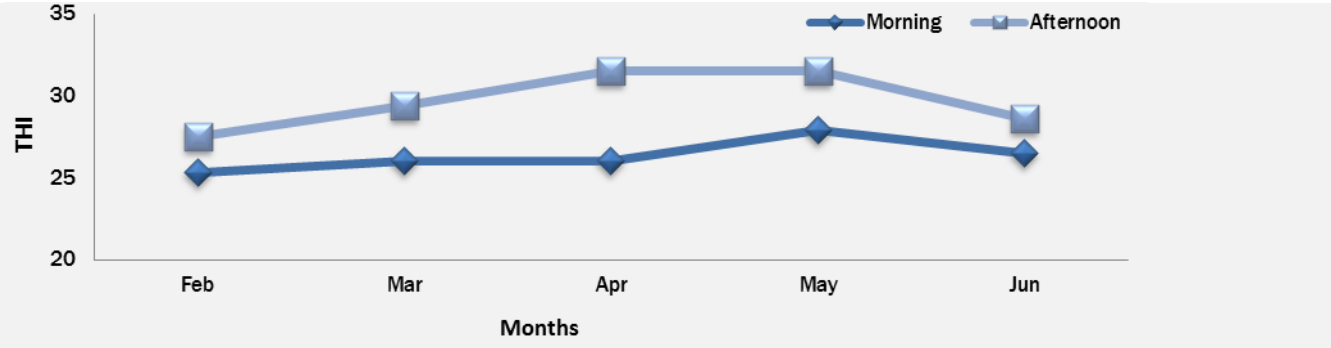

Figure 1 - Monthly temperature humidity index inside the rabbit house

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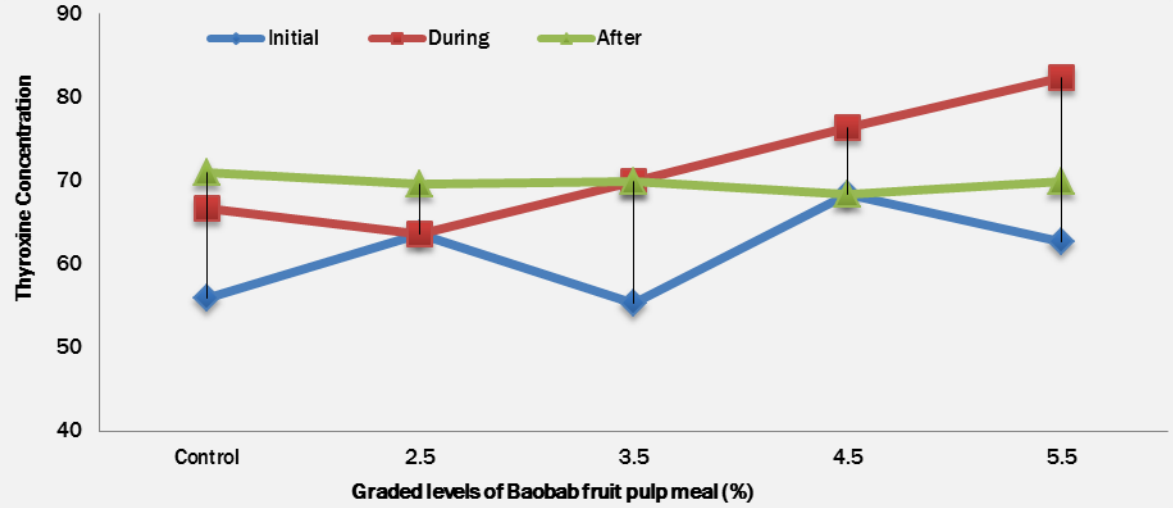

Figure 2 - Effect of graded levels of BFPM on Thyroxine concentrations (ng/ml) in adult rabbit does

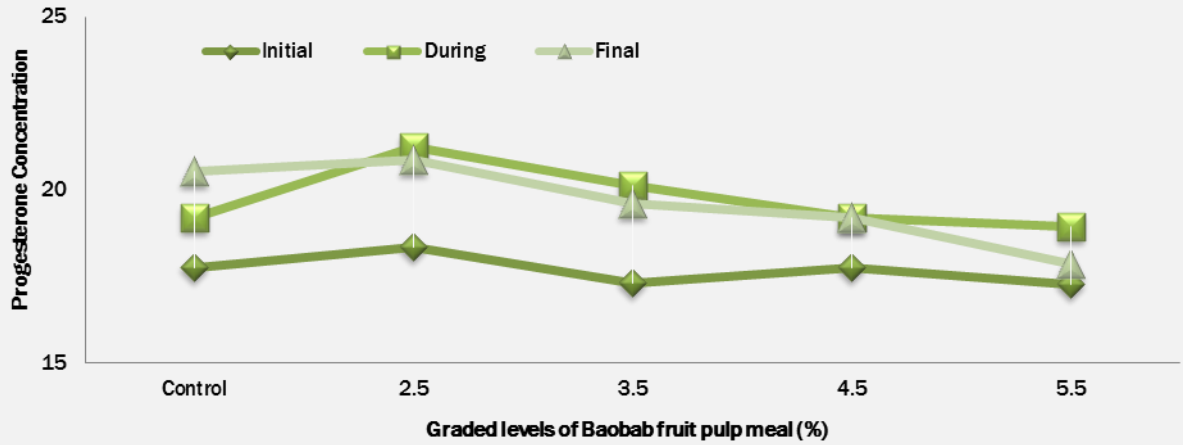

Figure 3 - Effect of graded levels of BFPM on progesterone levels ng/mL in adult rabbit does before, during and after pregnancy

Table 2 - Effect of graded levels of BFPM on serum metabolites of adult rabbit does

Parameters \ Treatments	Control	2.5%	3.5%	4.5%	5.5%	SEM
Before Pregnancy						
Glucose (mg/dl)	5.20	4.93	4.77	4.47	5.0	0.35
Total Protein (mg/dl)	63.00	66.67	66.00	67.67	67.33	1.43
Albumin (mg/dl)	36.07	35.67	39.67	37.67	37.33	1.12
Cholesterol (mg/dl)	1.57	1.53	1.47	1.47	1.53	0.11
Triglyceride (mg/dl)	0.93	1.00	0.90	0.87	0.67	0.10
Calcium (mg/dl)	2.33	2.40	2.27	2.56	2.29	0.07
Phosphorous (mg/dl)	1.05	1.02	1.16	1.13	1.01	0.06
During Gestation						
Glucose (mg/dl)	3.20^{b}	4.53^{a}	4.25^{a}	4.83^{a}	4.30^{a}	0.06
Total Protein (mg/dl)	68.33^{a}	62.00^{b}	67.00^{a}	65.00^{ab}	68.67^{a}	1.03
Albumin (mg/dl)	37.00	32.00	35.33	36.00	33.00	1.45
Cholesterol (mg/dl)	1.5	1.43	1.35	1.43	1.40	0.05
Triglyceride (mg/dl)	0.93	1.00	0.90	0.87	0.67	0.10
Calcium (mg/dl)	2.31^{ab}	2.37^{ab}	2.40^{a}	2.40^{a}	2.24^{b}	0.03
Phosphorous (mg/dl)	0.97^{ab}	0.93^{ab}	0.62^{b}	1.11^{a}	1.16^{a}	0.08
After Kindling						
Glucose (mg/dl)	4.57	5.33	4.50	4.00	4.40	0.14
Total Protein (mg/dl)	68.67^{a}	64.33^{c}	$68.00a^{b}$	$68.00a^{b}$	$65.00b^{c}$	0.79
Albumin(mg/dl)	42.00^{a}	35.33^{b}	40.33^{a}	39.17^{a}	34.33^{b}	0.84
Cholesterol (mg/dl)	1.43	1.50	1.40	1.35	1.50	0.04
Triglyceride (mg/dl)	$0.85b^{c}$	1.10^{a}	0.85^{b}	0.70^{c}	0.94^{ab}	0.04
Calcium (mg/dl)	2.17	2.31	2.43	2.41	2.18	0.02
Phosphorous (mg/dl)	1.11^{a}	1.0b	1.06^{a}	1.0^{b}	1.0^{b}	0.01

Means within rows with different superscript letters are significantly P < 0.05 different

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Table 3 - Effect of graded levels of BFPM on reproductive performance of adult rabbit does

Parameters \ Treatments	Control	2.5%	3.5%	4.5%	5.5%	SEM
Number of Parities/Doe	2	2	2	2	2	-
Gestation Period (days)	30	30	30	30.67	30.33	0.10
Average Litter size at birth	4.00^{bc}	3.67^{c}	5.66^{a}	5.33^{ab}	5.33^{ab}	0.23
Average Weight of litter (g)	186.67^{b}	176.67^{b}	233.33^{a}	218.33^{ab}	250.00^{a}	6.68
Average Weight of kitten (g)	47.33^{ab}	48.89^{a}	40.00^{b}	40.00^{b}	48.33^{a}	0.06
Average Litter size at weaning	3.67^{ab}	3.33^{b}	4.68^{ab}	5.00^{a}	5.00^{a}	0.29
Average Litter wt at weaning (g)	502.57	502.00	426.67	504.00	436.67	70.23

Means within rows with different superscript letters are significantly P<0.05 different

Table 4 - Physiological performance of Kittens

Parameters \ Treatments	Control	2.5%	3.5%	4.5%	5.5%	SEM
Rectal Temperature (C^{o})	38.51^{a}	37.80^{b}	37.62b^{c}	37.20^{c}	36.90^{d}	0.14
Heart Rate (beat/min)	140.44	139.16	139.94	135.75	136.10	2.25
Weight gain (g/day)	11.04	12.24	11.68	12.86	12.12	0.95

Means within rows with different superscript letters are significantly P<0.05 different

DISCUSSION

The Temperature humidity index (THI), an indicator of the thermal comfort level of the rabbits was calculated from the values of temperature and relative humidity readings in the pen house using the modified formula for the rabbit by Marai et al. (2001). The average THI value of the pen house was 29.5^{o}C and it is an indication that the rabbit house was severely thermally stressful and had adverse effects on the rabbits (Marai et al., 2001).

Heat stress negatively affected thyroxine secretion in this study (Figure 2.); this finding agrees with (Marai et al., 2004; Todini, 2017). BFPM supplemented groups had an increase thyroxine levels which may be due to the fact that vitamin C and other antioxidant have the ability to reduce oxidative stress and improve body metabolism (Ganaie et al., 2012; Sivakumar et al., 2010; Minuti et al., 2009). Vitamin C antioxidants alleviate retardation in thyroid functions, leading to the increase in serum thyroxine (Usha et al., 2002). Fetal demand for metabolites especially in the control group and 2.5% BFPM supplementation could have been responsible for the significant decrease of T_4 concentration in these treatments during pregnancy. Previous studies reported decrease in thyroid hormones with increasing temperature (Williams and Njoya, 1998; Sokolowicz and Herbut, 1999) decrease T_3 level and a concurrent increase in T_4 level (Marai et al., 2004) during heat stress. The values of thyroxin obtained after kindling is similar to the report of Mahmoud et al. (2014), Acclimation increases the adaptive ability of birds to subsequent thermal stress by reducing the level of T_3.

The trend in progesterone secretion noticed in this study may be attributed to the differences in the physiological status of the does and not necessarily due to heat stress. The effect of heat stress on plasma progesterone concentration is controversial (Marai et al., 2002; Anoh et al., 2018). In the present study, progesterone was low in the control, increased in 2.5% BFPM and decreases in 4.5% and 5.5% BFPM. However, heat stress may affect the number of mature follicles (Naseer et al., 2017) hence the decrease in progesterone secretion because of less number of *corpus luteum* sites as was noticed in the control. Vitamin antioxidants have been known to alleviate heat stress in rabbits (Prabsattroo et al., 2012, 2015; Anoh 2017; El-Desoky, et al., 2017). The presence of these phyto-chemical compounds may facilitate the ability of animals to rapidly grow follicles and shed more ova, creating more *corpus luteum* (CL) sites and increasing progesterone by counteracting free radicals and other reactive oxygen (ROS) species production in body fluids and tissues due to heat stress. The CL is a transient endocrine gland that secretes progesterone to support pregnancy in rabbits. The CL is maintained throughout the gestation (Theau-Cle´ment, et al., 1995) and P4 level in rabbits remain high throughout pregnancy period (Szendrő et al., 2010) a characteristic that differentiate the rabbits from other species.

Values of serum metabolites recorded in this result did not follow a particular pattern; however, serum metabolites were significantly high in BFPM treated groups. Ondruska et al. (2011) reported a decline in plasma TP with rising temperature. The general reduction in most of the serum metabolites during pregnancy compared to what was recorded after pregnancy agrees with the reports of Marai et al. 2007) During pregnancy, reductions in blood serum metabolite may be due to the decrease in feed intake of dams (Marai et al., 1994) and increase in water retention (Marai et al., 2004), and the high demand of the foetus at the late stages of pregnancy (Marai et al., 1994, 2004). The decrease in glucose in blood may be due to the increase in each glomerular filtration rate (GFR) (Marai et al., 1994) and foetal consumption and conversion of glucose to lactose of milk, and the decrease in each of renal threshold of glucose and capacity of renal tubules to absorb the glucose (Marai et al., 1994).

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Heat stress was responsible for the low litter size and conception rate recorded in the control and 2.5% BFPM. Heat stress was attributed to decrease follicular growth and number of ova that were shed as mentioned previously (Naseer et al., 2017). This might have affected conception rate hence reduced litter size. Fertilization failure or early embryonic mortality has been reported to be responsible for poor conception rate and small litter size (Argente, 2016). Heat stress was attributed in previous study for the decrease in receptivity and percentage of voluntary mating which led to a decline in fertility (Marai, et al., 2001) as was noticed in the control and 2.5% BFPM. The values of the litter weight were a reflection of the litter size of the rabbits. Treatments with large litter size had higher litter weight. The increase in the average weight of kits recorded in the control and 2.5% BFPM compared to 3.5% and 5.5% is because of their small litter sizes. Kitten in small litter had the opportunities to receive adequate breast milk compare to kittens in large litter. BFPM might have stimulated the increase in milk production which could have been responsible for the average weight of kitten noticed in 5.5% BFPM. Previous studies have demonstrated that feeding doe rabbits on diets containing vitamin antioxidants increases milk yield of does, litter size, and weight of litter (Argente, 2016; Fayeye and Ayorinde 2016).

The rectal temperature of kittens of BFPM treated does were low compared to the control. It is worthy to note that baobab contains high amount of vitamin C and other phyto-chemicals (Sena, 1998; PhytoTradeAfrica, 2009), antioxidant vitamin has been known to alleviate heat load in rabbits (In-Surk et al., 2014; Prabsattroo et al., 2012). The presence of these phyto-chemical compounds may facilitate the ability of animals to maintain their body homeostasis including body temperature by provoking endogenous cellular defense mechanisms to cope with oxidative stress and inflammation induced by heat stress (Akbarian et al., 2016). Heart rate and weight gain did not show any significant difference.

CONCLUSION

Heat stress reduced fertility in the does, litter size, hormone secretion and rectal temperature of kitten. Ameliorating heat stress with Baobab Fruit Pulp Meal (BFPM) improved reproductive performance, serum metabolites and hormonal concentrations. Baobab fruit pulp meal can be used up to 5.5% in reproductive diets of rabbits during hot periods.

DECLARATIONS

We declare that this research work is original and has not been published elsewhere.

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Authors' contribution
All authors (A. Kevin Usman, J. Nsisong Paul and U. Mary Amu) contributed equally in research and writing process.

Conflict of interests
The authors declare that there is no conflict of interests in this work.

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NUTRITIONAL VALUE AND *IN SITU* DEGRADABILITY OF SELECTED FORAGES, BROWSE TREES AND AGRO INDUSTRIAL BY-PRODUCTS

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ABSTRACT: The *in situ* dry matter and crude protein degradability of grasses, legumes, browse trees and agro industrial by products were evaluated by three fistulated bulls (Boran × Holstein-Friesian with mean body weight 580 kg and age= 29±3 months). The lower (P<0.05) crude protein content was reported in Bracharia grass than the other grasses. The higher (P<0.05) washing loss rapidly soluble nutrients (a) in Bracharia and Rhodes grasses and the greater (P<0.05) potential and effective degradability for dry matter (DM) and crude protein (CP) were observed in desho grass compared with the other grasses. The content of crude protein, relative feed value, washing loss or rapidly soluble nutrients (a) and potential DM degradability were higher (P<0.05) in Sesbania than Pigeon pea and tree lucerne browse trees. *Acacia nilotica* and Wanza (*Cordia African*) had the greater (P<0.05) washes loss (a), potential and effective degradability for dry matter and crude protein than the other browse trees. Cactus (*Cleistocactus sextoianus)* and Shola *(Ficus sure)* had the highest (P<0.05) undegradable protein than *Acacia nilotica* and Wanza (*Cordia african*). The energy source feed (maize bran) had the greater (P<0.05) potential and effective dry matter and crude protein degradability parameters than the other by products. The rumen undegradable protein was higher (P<0.05) in vetch than lablab. The rumen undegradable protein was higher (P<0.05) in Rhodes grass than the other forage grasses. Brewery spent grain and cotton seed cake have the higher (P<0.05) rumen undegradable protein than Noug seed *(Guizotia abyssinica)* cake, wheat and maize bran. The *in situ* dry matter and crude protein degradability values obtained in this study can be useful to identifying the best materials used for ruminant feeds.

Keywords: By-products, Dry matter, Forage, Rumen degradability, Bulls.

INTRODUCTION

The majority of ruminant animals in tropical Africa are raised on natural pastures which drop rapidly in quality (Smith et al., 1991; Amole et al., 2021). Many systems have been developed to predict the quality of forages fed to ruminants (Moore, 1994; Tedeschi et al., 2019; da Cruz et al., 2021). To parameterize the relative feed value system, the National Forage Testing Association selected equations that relate forage neutral detergent fiber and acid detergent fiber to dry matter intake and digestible dry matter with a base daily dry matter intake (DDMI) of 1.29% of daily body weight (Linn and Martin, 1989). Fluctuations in nutritional values result in very irregular growth and marked fluctuations in seasonal weights (Wilson, 1987). From understanding, and to a lesser amount from the extension of research results, small-scale farmers are increasingly relying on browse and by-products to supplement roadside grazing during the dry season (Odunlami, 1988; Duguma, 2020). Others animal feeds had poor degradability so that they may require some improvement before they can contribute to animal feed (Smith et al., 1988; Salami et al., 2019). This study was considered to evaluate the potential nutritive value of different animal feeds including forage, browse trees, and by-products commonly fed by ruminant animals.

MATERIALS AND METHODS

Study site and feed samples

The study was conducted at Holetta agricultural research center in animal nutrition research laboratory. The feeds including grass, legumes, browse trees, and agro industrial by products (protein and energy source concentrate feeds) were used for this study. The browse and agro industrial by-products animal feed samples were collected during the low rainy season while grasses and legumes forage feed samples were harvested and collected during the rainy season.

Chemical analysis

The green and fresh samples including grass and legume forages, browse trees and brewery spent grain were dried at 60 °C for 72 h and ground to pass through 1mm sieve size. The feed samples and residues after *In situ* dry matter degradability were analyzed through a standard procedure of AOAC (2005), this was used for dry matter, crude protein and ash content determination. The fiber fractions (neutral detergent fiber, acid detergent fiber and lignin) were analyzed by using the standard procedures (Van Soest and Robertson, 1985). Two-stage technique (Tilley and Terry, 1963) was employed to analyze *in vitro* digestibility dry matter the feed.

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In situ dry matter and crude protein degradability

The rumen degradability of the feeds was evaluated through (Orskov, 1982) procedure. All fee samples were ground to pass through 2mm sieve size. Duplicated feed samples were weighted (3 g) in a 6.5 × 14 cm nylon bag (50 µm pore size) and incubated in the rumens of three fistulated Boran × Holstein-Friesian bulls. The average body weight and age of experimental bulls were 580 kg and 29±3 months, respectively. The bulls were fed natural pasture hay (5.6% CP) *ad libitum* and about 2 kg concentrate feed (19.86% CP) bull^{-1} day^{-1} on dry mater basis. The bulls were offered the concentrate feed at every morning of 8 A.M. The bulls were housed in individual pens and provided water *ad libitum.* The bags with feed samples were incubated for 6, 12, 24, 48, 72, and 96 h. After removing the bag from rumen, it was washed in running water. Washing losses were determined in duplicate by weighing nylon bags with 3 g feed and then soaked in a tap water for about 30 minutes. The nylon bags were dried in oven at 60^{o}c for 72h and then weighed to determine the dry weight of the residues. Based on the following formula dry matter degradability was determined.

Dry matter degradability was calculated by $= \frac{((BW+S1)-(BW+RW))}{S1*DM} * 100$

Where: BW = Bag weight, RW = Residue weight, S1 = Sample weight, DM = Absolute dry matter of the original sample

Degradability (Y) of DM/CP was calculated by using the following equation

$Y = P = a + b (1 - e^{-ct})$, where:

a = soluble fraction

b = insoluble but potentially degradable fraction

c = degradation rate constant of the b fraction

t = degradation time (0, 6, 12, 24, 48, 72, and 96 h) and e = base for natural logarithm

Statistical analysis

The degradability parameters (a, b, and c) were estimated by using the general linear model procedures of statistical analysis, version 9.3 (Guide, 2010). Mean separation test was made using least significant differences analysis at P≤ 0.05. The linear model used was: Yij= µ + Fi + eij

where: Yij = response variable, µ = Overall mean, Fi = ith feed effect and eij = residual error.

Potential degradability (PD) for DM and CP was determined by the equation: PD = a + b,

Effective degradability (ED) for DM and CP was calculated through, ED = a + bc/k + c where: a = soluble fraction b = insoluble but potentially degradable fraction c = degradation rate constant of the b fraction k = rumen outflow rate (assumed to be 0.03/h). The effective degradability crude protein is similar to rumen degradable protein (RDP). The rumen undegradable protein (RUP) of each the sample was calculated as: RUP = 100 - RDP

RESULT AND DISCUSSION

Nutrient content and Relative feed value

The mean nutrient content of different animal feeds is presented Table (1). The higher (P<0.05) relative feed value and net energy content were obtained in Bracharia and Rhodes grasses compared with the other grasses. The lower (P<0.05) crude protein content was recorded in Bracharia grass than the other grass. Sesbania had the greater (P < 0.05) crude protein, relative feed value and net energy than the pigeon pea and tree lucerne browse forages. Vetch had a better (P<0.05) nutritional value than lablab. As compared with the other browse species, the higher (P<0.05) crude protein content and the better (P<0.05) relative feed value were observed in Wanza (*Cordia africana)* and cactus (*Cleistocactus sextoianus)*, respectively. Agam (*Carissa spinarum* L.) had the higher (*P*<0.05) net energy than Wanza (*Cordia africana)* and cactus (*Cleistocactus sextoianus*, but non-significantly different (*P* >0.05) with *Acacia nilotica*. Among concentrate feeds, Noug seed cake had the greater (p < 0.05) crude protein content and relative feed value than the other concentrate feeds.

Digestibility and fiber component

The average fiber fractions, digestibility and dry matter intake of grass and legume forages, browse species and agro industrial by product feed is presented in Table 2. The elephant grasses had the greater (P<0.05) acid detergent fiber (ADF), Acid detergent lignin (ADL) and Neutral detergent fiber (NDF) content than the other grasses. The *in vitro* dry matter digestibility, the calculated total digestible nutrient and dry matter intake were better (P< 0.05) in Bracharia and Rhodes grasses than elephant and desho grasses. Moreover, Rhodes grass had the higher (P< 0.05) dryb matter intake than desho grass. Sesbania had the lower (P<0.05) fiber fractions (ADF and NDF) compared with Pigeon pea and Tree lucerne browse legumes. Vetch had a better (P<0.05) *in vitro* dry matter digestibility and dry matter intake than lablab As compared with the other browse species, the least (P<0.05) fiber components in cactus (*Cleistocactus sextoianus* and the greater (P<0.05) total digestible nutrient in *Acacia nilotica* and Agam (*Carissa spinarum* L.) were reported in this finding. The higher (P<0.05) neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL) content were reported in brewery spent grain and cotton seed cake than the other agro industrial by products. Noug (*Guizotia abyssinica*) seed cake and wheat bran have the greater (P<0.05) *in vitro* dry matter digestibility, calculated total digestible nutrient and dry matter intake compared with the other agro industrial by products.

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Table 1 - Nutrient content and relative feed value of grass and legume forages, browse species and by product feeds

Feeds		Nutrient Parameters (%, DM basis)				
		DM	Ash	CP	RFV	NE1 (Mcal Kg^{-1})
Forage Grasses	Bracharia grass	92.92^{a}	8.92^{b}	7.35^{b}	90.19^{a}	1.45^{a}
	Desho grass	90.82^{d}	13.06^{a}	9.85^{a}	81.62^{b}	1.30^{b}
	Elephant grass	91.53^{b}	11.74^{a}	8.78^{a}	74.36^{c}	1.24^{c}
	Rhodes grass	91.16^{c}	12.66^{a}	9.96^{a}	92.37^{a}	1.45^{a}
	SE	0.09	0.51	0.35	1.07	0.01
	CV	0.19	8.80	8.01	2.54	1.94
	LSD	0.28	1.63	1.12	3.44	0.04
Browse Legumes	Pigeon pea	92.58	13.39^{a}	20.55^{c}	108.01^{b}	1.33^{b}
	Sesbania	91.96	10.14^{b}	30.81^{a}	224.02^{a}	1.67^{a}
	Tree lucerne	91.20	5.56^{c}	25.55^{b}	100.19^{b}	1.40^{b}
	SE	0.21	1.13	0.53	17.74	0.05
	CV	0.43	7.91	3.92	24.16	5.90
	LSD	0.51	0.67	0.45	3.24	0.01
Forage Legumes	Vetch	93.16^{a}	14.57	28.35^{a}	116.52^{b}	1.41
	Lablab	91.10^{b}	14.28	22.55^{b}	120.58^{a}	1.40
	SE	0.21	1.13	0.53	17.74	0.05
	CV	0.43	7.91	3.92	24.16	5.90
	LSD	0.51	0.67	0.45	3.24	0.01
Browse Trees	*Acacia nilotica*	90.63^{bc}	2.25^{e}	14.78^{b}	188.03^{b}	1.89^{ab}
	Agam (*Carissa spinarum* L.)	90.20^{c}	8.75^{d}	8.34^{c}	167.53^{bc}	1.90^{a}
	Cactus (*Cleistocactus sextoianus)*	91.00^{b}	19.72^{a}	8.92^{c}	359.46^{a}	1.86^{b}
	Wanza (*Cordia african)*	93.23^{a}	11.84^{b}	22.83^{a}	191.70^{b}	1.64^{c}
	Shola *(Ficus sure)*	93.37^{a}	10.15^{c}	15.85^{b}	142.33^{c}	1.63^{c}
	SE	0.16	0.30	0.45	9.54	0.01
	CV	0.31	4.87	5.45	7.88	1.07
	LSD	0.53	0.97	0.59	31.12	0.4
By products	Brewery grain	23.17^{b}	4.23^{c}	25.43^{c}	96.77^{d}	1.57^{c}
	Cotton seed cake	90.55^{a}	4.99^{b}	29.51^{b}	117.82^{c}	1.34^{d}
	Maize Bran	91.31^{a}	5.39^{b}	11.06^{e}	192.05^{b}	1.99^{a}
	Noug seed cake	91.31^{a}	8.04^{a}	34.08^{a}	280.71^{a}	1.87^{b}
	Wheat bran	90.6^{a}	4.00^{c}	16.60^{d}	206.66^{b}	2.01^{a}
	SE	0.77	0.16	0.44	4.96	0.03
	CV	1.73	5.26	3.18	4.81	2.51
	LSD	2.52	0.53	1.45	16.18	0.08

Mean values in the columns without common superscripts are significantly different at (P<0.05), DM= dry matter, CP = Crude protein, RFV =Relative feed value, NE = Net energy, SE= standard error, CV= coefficient variation and LSD =least significance differences

Table 2 - Means of fiber fraction, digestibility (%, DM basis) and dry matter intake (g Kg^{-1} of body weight) of forages browse and by product feeds

Feeds		Fiber fractions and other components (%, DM basis)					
		ADF	ADL	NDF	IVDMD	TDN	DMI
Forage Grasses	Bracharia grass	32.34^{c}	3.88^{c}	65.71^{bc}	62.26^{a}	59.61^{a}	18.3^{ab}
	Desho grass	38.39^{b}	4.76^{b}	67.26^{b}	63.08^{a}	51.79^{b}	17.8^{b}
	Elephant grass	40.72^{a}	5.17^{a}	71.54^{a}	46.72^{c}	48.79^{c}	16.8^{c}
	Rhodes grass	32.59^{c}	4.11^{c}	63.96^{c}	51.35^{b}	59.29^{a}	18.8^{a}
	SE	0.50	0.13	0.66	1.17	0.64	1.23
	CV	2.76	5.69	1.98	4.22	2.34	1.99
	LSD	1.59	0.41	2.12	3.77	2.05	0.5
Browse Legumes	Pigeon pea	36.96^{a}	8.32	51.77^{a}	55.28^{c}	53.63^{b}	23.2^{b}
	Sesbania	24.11^{b}	7.16	31.32^{b}	66.27^{a}	70.23^{a}	40.7^{a}
	Tree lucerne	34.37^{a}	8.01	57.70^{a}	61.64^{b}	56.97^{b}	20.8^{b}
	SE	1.64	0.37	2.87	1.10	2.13	2.62
	CV	9.23	10.44	11.45	3.53	7.06	18.59
	LSD	1.45	0.32	1.23	2.14	4.67	5.67
Forage Legumes	Vetch	34.09	6.78	49.90^{a}	58.06^{b}	57.34	24.1
	Lablab	34.54	7.07	47.86^{b}	61.40^{a}	56.76	25.1
	SE	1.64	0.37	2.87	1.10	2.13	2.62
	CV	9.23	10.44	11.45	3.53	7.06	18.59
	LSD	1.45	0.32	1.23	2.14	4.67	5.67
Browse Trees	*Acacia nilotica*	17.30^{bc}	9.69^{a}	37.32^{c}	58.20^{a}	79.02^{ab}	32.1^{bc}
	Agam (*Carissa spinarum* L.)	16.57^{c}	10.67^{a}	42.20^{b}	50.75^{c}	79.96^{a}	28.5^{cd}
	Cactus (*Cleistocactus sextoianus)*	18.43^{b}	4.02^{d}	26.40^{e}	53.13^{bc}	77.56^{b}	62.2^{a}
	Wanza (*Cordia african)*	26.71^{a}	8.45^{b}	33.03^{d}	55.59^{b}	66.86^{c}	36.4^{b}
	Shola *(Ficus sure)*	26.72^{a}	6.93^{c}	44.49^{a}	58.43^{a}	66.86^{c}	26.9^{d}
	SE	0.37	0.34	0.60	0.78	0.48	1.44
	CV	3.05	7.33	2.93	2.45	1.12	6.68
	LSD	1.21	0.34	1.45	1.11	1.57	4.68
By products	Brewery grain	27.93^{b}	6.43^{a}	64.63^{a}	63.10^{c}	65.29^{c}	18.57^{d}
	Cotton seed cake	36.72^{a}	6.05^{a}	47.65^{b}	51.35^{d}	53.94^{d}	25.20^{c}
	Maize Bran	12.12^{d}	3.16^{b}	38.50^{c}	76.19^{b}	85.73^{a}	31.17^{b}
	Noug seed cake	16.38^{c}	4.14^{b}	25.26^{d}	79.46^{a}	80.20^{b}	47.57^{a}
	Wheat bran	10.97^{d}	3.16^{b}	36.17^{c}	82.01^{a}	87.18^{a}	33.17^{b}
	SE	1.00	0.33	0.92	0.90	1.29	0.7
	CV	8.33	12.66	3.73	2.20	3.01	3.93
	LSD	3.01	1.10	2.98	2.92	2.52	18.28

Mean values in the columns without common letters are significantly different at (P<0.05), NDF= Neutral detergent fiber, ADF= Acid detergent fiber, ADL= Acid detergent lignin, IVDMD=Invitro dry matter digestibility, TDN= Total digestible nutrient, DMI= dry matter intake, SE= standard error, CV= coefficient variation and LSD =least significance differences.

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Dry matter degradability

The mean dry matter degradability of different grass, legume, browse species and agro industrial by product feeds is presented in Table 3. The washing loss (a) was higher (P<0.05) in Bracharia and Rhodes grasses than the other grasses but the higher (P<0.05) potential and effective DM degradability was observed in desho grass compared to other grasses. As compared with the other browse legumes, the lower (P<0.05) washing loss and the higher potential and effective DM degradability (P<0.05) were recorded in Pigeon pea and tree lucerne, respectively. Sesbania had the higher potential DM degradability while Pigeon pea and tree lucerne had the lowest values, meaning that the amount of dissolved material in Sesbania was the highest. *Acacia nilotica* and Wanza (*Cordia africana*) browses have the higher (P<0.05) washes loss, potential and effective degradability than the other browse species. Among concentrate feeds, maize bran and wheat bran had he superior (P<0.05) water wash fraction, potential and effective dry matter degradability than the other concentrate feeds.

Table 3 - Ruminal dry matter degradation kinetics of different animal feeds

Feeds		Parameters (%, DM basis)				
		a	b	c	PD	ED
Forage Grasses	Bracharia grass	10.41^{a}	45.55^{b}	0.030^{bc}	55.96^{b}	33.38^{b}
	Desho grass	7.73^{b}	56.83^{a}	0.037^{ba}	64.56^{a}	38.97^{a}
	Elephant grass	7.22^{b}	44.25^{b}	0.038^{a}	51.47^{c}	32.03^{b}
	Rhodes grass	9.64^{a}	40.77^{c}	0.028^{c}	50.42^{c}	29.45^{c}
Browse Legumes	Pigeon pea	8.20^{c}	31.46^{c}	0.06^{b}	39.66^{b}	28.91^{b}
	Sesbania	21.19^{a}	46.58^{b}	0.07^{b}	67.77^{a}	52.62^{b}
	Tree lucerne	10.39^{b}	56.06^{a}	0.10^{a}	66.45^{a}	53.48^{a}
Forage Legumes	Vetch	25.07^{a}	35.79^{b}	0.08^{b}	60.86^{b}	51.13^{b}
	Lablab	21.41^{b}	52.53^{a}	0.13^{a}	73.93^{a}	63.87^{a}
Browse Trees	*Acacia nilotica*	9.15^{a}	75.70^{a}	0.05^{b}	84.85^{a}	57.00^{a}
	Agam (*Carissa spinarum* L.)	9.69^{b}	53.16^{cd}	0.07^{a}	62.84^{c}	46.40^{b}
	Cactus (*Cleistocactus sextoianus)*	8.82^{b}	50.52^{d}	0.05^{b}	59.35^{d}	41.39^{c}
	Wanza (*Cordia africana*)	11.96^{a}	61.16^{b}	0.04^{c}	73.12^{b}	47.44^{b}
	Shola (*Ficus sure)*	8.58^{b}	55.54^{c}	0.04^{c}	64.12^{c}	41.43^{c}
By products	Brewery grain	8.82^{e}	60.05^{c}	0.07^{b}	67.87^{d}	42.96^{e}
	Noug seed cake	9.27^{d}	64.18^{b}	0.24^{a}	73.45^{bc}	66.10^{c}
	Cotton seed cake	14.94^{c}	56.55^{c}	0.03^{b}	71.49^{c}	43.57^{d}
	Wheat bran	21.05^{b}	54.11^{c}	0.27^{a}	75.16^{b}	69.60^{b}
	Maize bran	26.64^{a}	70.66^{a}	0.07^{b}	97.30^{a}	75.02^{a}

Mean values in the columns without common superscripts are different at (P<0.05): a = soluble fraction, b = insoluble but potentially degradable fraction c = degradation rate constant of the b fraction, PD= Potential degradability and ED= Effective degradability (at 0.02)

Crude protein degradability

The average crude protein degradability of grass and legume forages, browse species and by product feeds was significantly affected by type and is presented in Table 4. The washing loss fraction (a) of crude protein was higher (P<0.05) in Bracharia and Rhodes grasses than the other grasses but the upper (P<0.05) potential and effective degradability of crude protein was observed in desho and Bracharia grasses. The desho and elephant grass have a smaller soluble CP fraction than Bracharia and Rhodes grasses. The rumen undegradable protein was better (P<0.05) in vetch (30.4%) than lablab (24.98%) forage. The rumen undegradable protein of Pigeon pea and Sesbania was increased by 23-26% than the rumen undegradable protein of tree lucerne. *Acacia nilotica* and Wanza (*Cordia africana*) browse species had the higher (P<0.05) crude protein potential and effective degradability than the other browse species. The maize bran and wheat bran had the greater (P<0.05) soluble nutrient fraction, potential and effective crude protein degradability than the other concentrate feeds.

In line with earlier finding by Hadjipanayiotou and Economides (2001) vetch had the highest CP content (28.35% DM basis) whereas the CP content of lablab relatively closes (22.55% DM basis). The low digestibility and dry matter intake of the feed could be attributed by the higher fiber components which might be limited a microbial access to digest feed and fiber content. The small amount of soluble DM fraction in desho and elephant grass is supported with the previous result of Kabi et al. (2005). The Sesbania browse have the greater potential dry matter degradability value while Pigeon pea and tree lucerne had the lowest values, meaning that the amount of dissolved material and the degradable components in Sesbania was the highest but this value is relatively lower as compared with the other report (Rahmat and Permana, 2021), the potential and effective dry matter degradability of vetch in this study is comparable with the previous study (Hadjipanayiotou and Economides, 2001). The potential and effective degradability of DM and CP in *Acacia nilotica* and Wanza (*Cordia africana*) browse species in this study in agreement with the previous report (Rahmat and Permana, 2021). In this study desho and elephant grass have a smaller soluble CP fraction than Bracharia and Rhodes grasses,

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which is comparable with the other finding (Kabi et al., 2005). Comparable potential and effective crude protein degradability in the vetch has been reported in earlier (Hadjipanayiotou and Economides, 2001).

Table 4 - Ruminal crude protein degradation kinetics of different feeds

Feeds		Parameters (%, DM basis)					
		a	b	c	PD	ED	RUP
Forage Grasses	Bracharia grass	5.14^{a}	45.15^{b}	0.031^{b}	50.29^{b}	27.86^{b}	72.14^{b}
	Desho grass	1.04^{b}	56.43^{a}	0.037^{a}	57.47^{a}	32.24^{a}	67.76^{c}
	Elephant grass	1.72^{b}	44.25^{b}	0.038^{a}	45.97^{c}	26.53^{b}	73.47^{b}
	Rhodes grass	4.14^{a}	40.77^{c}	0.028^{b}	44.92^{c}	23.95^{c}	76.05^{a}
Browse Legumes	Pigeon pea	0.82^{c}	58.05^{b}	0.07^{b}	58.87^{c}	40.96^{c}	59.04^{a}
	Sesbania	14.94^{a}	56.55^{b}	0.03^{b}	71.49^{b}	43.57^{b}	56.43^{a}
	Tree lucerne	9.27^{b}	64.18^{a}	0.24^{a}	73.45^{a}	66.10^{a}	33.90^{b}
Forage Legumes	Vetch	21.05^{b}	54.11^{b}	0.27^{a}	75.16^{b}	69.60^{b}	30.40^{a}
	Lablab	26.64^{a}	70.66^{a}	0.07^{b}	97.30^{a}	75.02^{a}	24.98^{b}
Browse Trees	*Acacia nilotica*	7.23^{b}	75.46^{a}	0.05^{b}	82.69^{a}	54.82^{a}	45.18^{c}
	Agam (*Carissa spinarum* L.)	7.49^{b}	53.16^{cd}	0.07^{a}	60.64^{c}	44.20^{a}	55.80^{b}
	Cactus (*Cleistocactus sextoianus)*	6.62^{b}	50.52^{d}	0.05^{b}	57.15^{d}	39.19^{c}	60.81^{a}
	Wanza (*Cordia africana*)	9.76^{a}	61.16^{b}	0.04^{c}	70.92^{b}	45.24^{b}	54.76^{b}
	Shola (*Ficus sure)*	6.52^{b}	55.46^{c}	0.04^{c}	61.98^{c}	39.32^{c}	60.68^{a}
By products	Brewery grain	7.38^{d}	41.68^{c}	0.04^{b}	48.08^{e}	29.73^{d}	70.27^{a}
	Noug seed cake	5.33^{c}	58.28^{b}	0.22^{a}	63.60^{c}	56.40^{c}	43.60^{b}
	Cotton seed cake	1.95^{d}	56.08^{b}	0.02^{b}	57.03^{d}	24.20^{d}	75.80^{a}
	Wheat bran	21.06^{b}	52.79^{b}	0.26^{a}	73.85^{b}	68.33^{b}	31.67^{c}
	Maize bran	25.64^{a}	70.66^{a}	0.07^{b}	96.30^{a}	74.02^{a}	25.98^{c}

Mean values in the columns without common superscripts are different at (P<0.05: a = soluble fraction, b = insoluble but potentially degradable fraction, c = degradation rate constant of the b fraction, PD= Potential degradability, ED= Effective degradability (at 0.02) and RUP=Rumen undegradable protein

CONCLUSION

In the result of the current study the different grass and legume forages, browse legumes and trees as well as agro industrial by-products have good nutritional value. The *in situ* dry matter degradability and rumen undegradable protein of the studied feeds can be useful to predict the highest materials used for ruminant feeds. Among the studied forage feeds Rhodes grass, Pigeon pea, Sesbania, vetch, cactus (*Cleistocactus sextoianus)* and brewery by products had the higher rumen undegradable protein than the other animal feeds and recommended for ruminant feeds.

DECLARATIONS

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Data availability

The data used to support the findings of this study are available from the corresponding author on reasonable request.

Authors' contributions

Conceptualization, investigation methodology, and writing - original draft was done by G. Terefe; data cleaning and data analysis by SAS software was done by M. Faji; formal analysis and writing, review and editing of the paper was done by G. Mengistu.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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We suggest the following format (please use initials to refer to each author's contribution): AB carried out the molecular genetic studies, participated in the sequence alignment and drafted the manuscript. JY carried out the immunoassays. MT participated in the sequence alignment. ES participated in the design of the study and performed the statistical analysis. FG conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript. For authors that equally participated in a study please write '**All/Both authors contributed equally to this work**.' Contributors who do not meet the criteria for authorship should be listed in an acknowledgements section.

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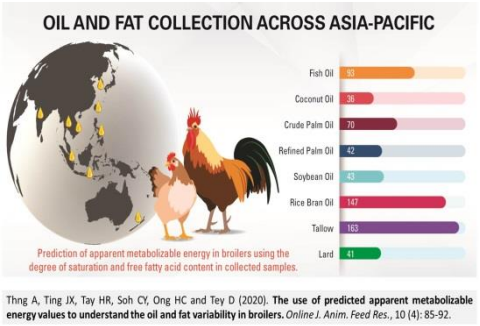

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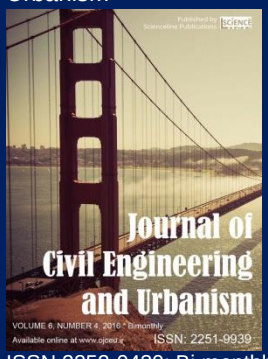

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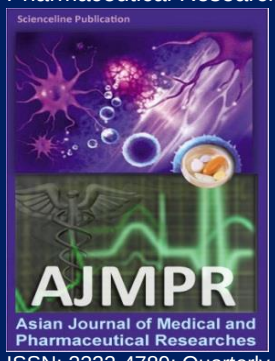

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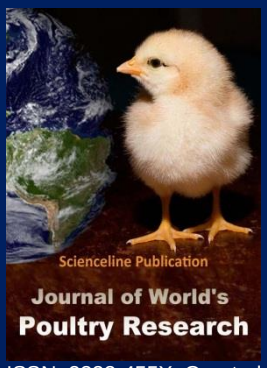

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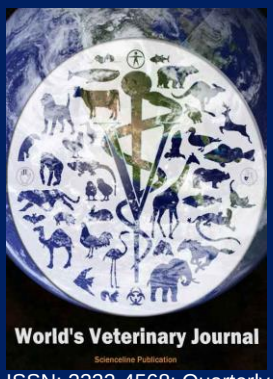

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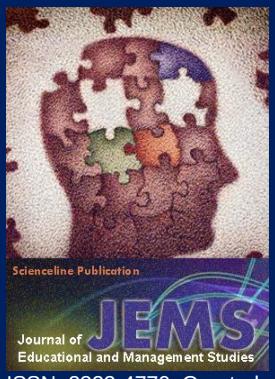

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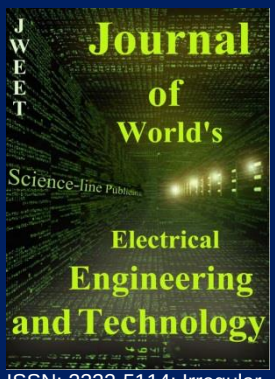

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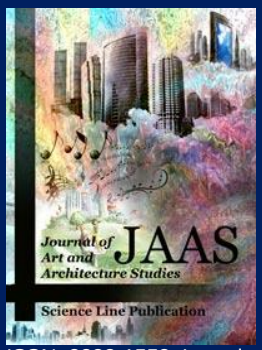

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