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Review article

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Review article

CATTLE TEMPERAMENT AND REPRODUCTIVE SUCCESS

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Cattle temperament is defined as the fear-related behavioral responses when exposed to human handling. As cattle temperament worsens, their response to human contact or any other handling procedures becomes more pronounced. The agitated and/or aggressive response expressed by cattle with excitable temperament when exposed to human handling can be attributed to their fear and consequent inability to cope with this situation. In addition to altered behavior, temperamental cattle may also experience changes in their body physiology, and the hormones produced during this fear-related stress reaction influence several factors, such as growth and health.Within the cattle industry, producers select cattle for temperament, primarily for safety reasons. However, our recent studies demonstrate cattle temperament may also have productive and economic implications to beef operations. Stress increases stress hormones such as cortisol levels and results in lowering the levels of reproductive hormones. Most cattlemen recognize that cattle under stress may not eat as well but may not know of its effect on reproductive behavior and physiology. This article will summarize our findings that could have an impact on the beef herd.

Cattle temperament assessment methods

A number of techniques have been used to assess cattle temperament, both subjective and objective in nature, with varying positive and negative attributes. At the farm level, if the goal is to base management decisions on temperament, it is crucial that these methods are easy to implement and consistent. Two of the most frequently used techniques for assessing beef cattle temperament, most likely due to their ease of execution, are chute temperament score and chute exit speed. Chute exit speed and gait methods with 5- or 6-point scales have been used to determine effects of temperament on production and reproduction. Cattle that are assigned a score of 1 or 2 on this scale are considered to be docile in temperament, and those assigned a 4 or 5 are considered to be excitable. Studies investigating the relationship between temperament assessment techniques most often look at the correlation between chute temperament score and chute exit speed. In general, results indicate a low to moderate positive correlation between chute temperament score and chute exit speed following a one-time assessment. Additional tests, including the Movement Measuring Device (MMD) and calculating exposed eye white percentage (EW) are being investigated as potential temperament assessment techniques. The MMD is used to

quantify the amount of movement made by an animal while restrained in a weigh scale, and is theorized to provide more information about the force and frequency of movement of restrained cattle. One of the first studies looking at exposed EW as a measure of temperament in beef cattle. In regard to EW,moderate to high positive correlations between exposed EW percentage and chute temperament score, and low to moderate correlation between exposed EW and a form of chute exit speed. The technique used to determine EW was reported to be highly repeatable and accurate, as multiple images per animal were analyzed twice and found to be highly, positively correlated.

In our studies, the 5- or 6-point scoring system was modified to a 2-point scoring method: **calm -** cattle that exited the chute slowly and walked towards herd mates; and **excited** - cattle that exited the chute jumping, trotting, or running towards herd mates.

Effect of temperament on Reproductiion

In our first study, beef heifers at eight different ranches were scored for temperament by using "chute exit and gait" as a measure of excitability [1]. The scoring system was: 0 = calm (slow exit and walk); 1 = excitable (fast exit and jump or trot or run). The scoring was done at the time of initiation of synchronization program for artificial insemination (AI) along with taking a blood sample for cortisol, progesterone and substance-P (associated with the regulation of stress and anxiety). The percentage of heifers with excitable temperaments varied across the eight locations from 21 to 44%. The key outcome was that temperament affected AI pregnancy rates. The AI pregnancy rates differed between heifers with excitable (51.9%) and calm temperament (60.3%). Another important finding was that the design of the cattle-handling facility also had an effect on temperament and consequently on AI pregnancy rate. Ranches with chute area alley-ways that had acute bends and turns or long straight alley-ways had lower AI pregnancy rates (53.5%, 56.3%) compared to those with a semicircular alley-way (67.0%). Handling facilities with alley-ways with acute bends and turns had a greater proportion of excitable heifers and higher stress hormones in their heifers.

In a subsequent study with two experiments, we looked at the effect of beef cow temperament on reproductive performance [2]. In the first, cows on eight ranches were evaluated for temperament using chute-exit and gait scores (as above), as well as body condition, before being turned out with the bulls. They were examined for pregnancy by palpation 35 days after the end of the 85-day breeding season. Cows with an excitable temperament took 24 more days to become pregnant compared to calm cows (median days to pregnancy, 35 vs 59 days). In the second experiment, cows were managed similarly but checked for pregnancy at 2 and 6 months after the end of the breeding season. Controlling for body condition, pregnancy loss was greater in excited versus calm cows (5.5 versus 3.2% loss).

In another study that looked at embryo transfer success, the team found that cows with a calm temperament had higher pregnancy rates after embryo

transfer compared to cows that were excitable (59 versus 52%) [3]. The study also compared cows given a non-steroidal anti-inflammatory drug injection or not at the time of embryo transfer. Excitable cows that were not treated with anti-inflammatory drug had lower pregnancy rates by 10% as well as lower progesterone and higher cortisol levels than calm cows. The calm cows' pregnancy rate was similar whether treated or not.

In a study published in 2018, they looked at factors that influenced calf temperament at weaning and the association of weaning temperament and temperament at breeding and effect on heifer AI pregnancy rate at three locations [4]. They also looked at cow age and the calf's sire docility EPD (the likelihood that the calf will inherit genes for acceptable behavior -- a greater docility EPD is associated with offspring exhibiting calmer behavior). Calves were observed for behavior from 1 to 4 weeks (nursing, play, interaction with cow and other calves, altered gait and resting) and treatment records were reviewed.

About half (51%) of the cows and 39% of the calves were classified as excitable. Factors that were associated with excitable calves included their dam's temperament, a reduction in play, illness, altered gait and sire docility EPD. A low sire docility EPD and cow excitability were associated with a greater likelihood for excitability in the calf. Circulating cortisol concentrations were greater in excitable females compared to calm females prior to weaning. Excitable temperament of calves at weaning had a greater likelihood of excitable temperament as heifers at breeding. The calf's excitable temperament at weaning was associated with lower pregnancy to AI.

This research captures a pretty clear picture that cattle temperament has an effect on beef cow and heifer reproduction. Although temperament can be influenced by many factors, there is strong evidence that genetics plays a role and that beef producers have control over their herd's genetics. Another factor under their control is the design of their handling facilities as these too can influence the handling stress that cattle have to endure. Stress effects cortisol levels which can affect important reproductive hormone levels, leading to a better understanding of conception failure and pregnancy loss and why we should minimize stress when handling cattle.

In summary, excitable temperament is a fear-related behavioral response that has detrimental effects on the performanceof cattle. This research provides evidences that cattle temperament has an effect on beef cow and heifer reproduction. Producers can evaluate cattle temperament by visual assessments that can be conducted during common handling procedures. Depending on the outcomes, producers can adopt management strategies to improve the overall temperament of the cow herd. Although temperament can be influenced by many factors, there is strong evidence that genetics plays a role and that beef producers have control over their herd's genetics. Another factor under their control is the design of their handling facilities as these too can influence the handling stress that cattle have to endure. Other factors are early age acclimation to human handling and consideration of temperament in selection/

culling decisions. This will bring benefits to cattle performance and the consequent productivity of beef operations containing temperamental cattle.

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Full Length Articles

THE PRELIMINARY PHYTOCHEMICAL SCREENING OF VARIOUS LEAF EXTRACTS OF PLANT *Limonia acidissima Linn*.

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ABSTRACT

The plant Limonia acidissima belongs to family Rutaceae. Various parts of this plant like fruit, seed, rind, bark, leaves and roots are being used to treat human and animal ailments. The fruits of the plant are most essential therapeutic portion containing bioactive molecules. This study is intended to reveal presence of some phytoconstituents from leaf extracts and review it thoroughly in order to validate its medicinal use. In the study it was found that aqueous and ethanolic extracts revealed maximum types of phytochemical classes as compared to acetone and petroleum ether extract which can be used for treatment with reference to its mentioned use. Many phytochemicals namely alkaloids, carbohydrates, glycosides, proteins, saponins, phenols, resins, etc. were detected which reflects its therapeutic value.

Key words: *Limonia acidissima*, leaf extract, phytochemical investigation

INTRODUCTION

The plants are being used in therapeutics across the globe since time immemorial. Even in current day situation man is looking for alternative therapies with plant as a source for newer phytomolecules. It is interesting to mention that significant population of the world still relies on plant medicines. India is home for millions of plant species with some famous for their medicinal properties and some still unexplored.

The fruits of the plant *Limonia acidissima* popularly called wood apple, or elephant apple are known as poor mens food. The fruit pulp of the plant possesses antidiabetic, wound healing, anticancer, astringent, antioxidant, hepatoprotective, antifungal, antispermatogenic activity, dyspepsia, stomachic activity (Vijayvargia and Vijayvargia, 2014, Pandey *et al*., 2014, Deshpande D J., 2006). Studies by some authors suggest fruit to bear promising antibacterial activity with dose dependent inhibition (Jayashree and Londonkar, 2014, Ponnuraj *et al*., 2015, Pandey *et al*., 2014). The leaves are mentioned to possess antibacterial activity, antilarvicidal, antioxidant, astringent, antifungal, diuretic activity, various types of gastropathy and cardiopathy (Vijayvargia and Vijayvargia, 2014, Prajapati *et al*., 2009, Deshpande D

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J., 2006, Naidu *et al.*, 2014, Attarde *et al.,* 2011). Many authors worked on antibacterial activity of leaves and results varied since some author given good remarks (Naidu *et al*., 2014, Panda *et al*., 2013), but some claims poor antbacterial activity (Patil *et. al.,* 2012, Thomas and Ponnammal, 2005). The plant has ethno-veterinary medicinal importance in Vidarbha region of Maharashtra, since tribals use leaves of tree against haemorrhagic septicaemia (Kulkarni *et al.*, 2014) in bovines. The leaves possess tannins and essential volatile oils (Vijayvargia and Vijayvargia, 2014, Prajapati *et al*., 2009, Nadkarni A. K., 1954). The active principles of the plants *Limonia acidissima* were known to be mostly residing in whole fruit and hence researcher were concentrated towards biological utility and pharmacological activity of only fruits. But, other parts of this plant including leaf remained untouched. Hence, taking into consideration its age old traditional medicinal use and ethnoveterinary use, the study on leaves is conducted to reveal certain phytochemicals.

MATERIALS AND METHODS

Materials – Distilled water, analytical grade solvents like ethanol, acetone and petroleum ether provided by Hi-Media, musclin cloth, Whatman filter paper number 1. The chemicals used for phytochemical analysis were all analytical grade.

Collection, identification and authentication of plant: The collection of plant material was done in the month of August. The branches possessing fresh green young to mature leaves were selected and submitted to Botany department of Rashtrasant Tukadoji Maharaj Nagpur University. The plant was identified as *Limonia acidissima* and herbarium sheet with appropriate information regarding identified plant species were filled up by an expert botanist and given authentication number **10056**. One herbarium sheet was deposited in the respective department for future reference.

(1) Preparation of extract - The extraction was done as per the methodologies employed by **Betoni *et al.*, (2006)** and **Mahomoodally** and **Dilmohamed (2016)** with very slight modifications. The extraction of leaves was done by cold exhaustive maceration method. The collected plant leaves were air dried under shed at normal room temperature. Dried leaves were subjected to grinding to a coarse powder form. Then 100 gm leaf powder was transferred into a stoppered round bottom long neck flask and added 400 ml of solvent. The plant powder was allowed to macerate for 24 hrs at room temperature with vigorous intermittent shaking of the flasks for 10 - 15 minutes after every 2 - 3 hrs to facilitate or accelerate the extraction process. The contents present in flask were taken out and filtered through muslin cloth with care that complete filtrate was obtained. The collected filtrate was again subjected for further filtration by Whatman filter paper no. 1. The final filtrate was collected in a petridish weighed previously. The marc remained after filtration in above process was again added with 400 ml solvent and allowed to macerate for next 24 hrs. Similar process was repeated for third time till complete decolorization of marc occurred and filtrates obtained through second and third maceration process were combinely

evaporated on hot water bath at 50ºc with previously obtained filtrate. Finally solvent free, semi solid extract was obtained.

The entire procedure was performed with four different solvents like water, ethanol, acetone and petroleum ether. The extracts thus obtained were stored in dessicator for further use. The percentage extractability has been calculated from above information for each individual extract.

(2) Qualitative Phytochemical analysis - The preliminary qualitative phytochemical analysis was done to detect presence or absence of various phytoconstituents in given leaf extracts of *Limonia acidissima* as per method described by **Rosenthaler (1930)** and **Raaman (2006).** The test revealing negative results were performed twice for confirmation.

1. Test for Alkaloids

Fifty milligrams of extract was stirred with dilute hydrochloric acid and filtered. The filtrate was then subjected to following tests. (A) Mayer's test

Few millilitres of filtrate were taken in a test tube and few drops of Mayer's reagent were added along side wall of test tube. Formation of white or creamy precipitate indicates positive test.

Mayer's reagent: Mercuric chloride (1.358 grams) was dissolved in 60 millilitres of water and potassium iodide (5 grams) was dissolved in 10 millilitres of water. These two solutions were mixed and volume was made up to 100 millilitres with water.

(B) Wagner's test (Rosenthaler, 1930)

To the little amount of filtrate, Wagner's reagent was added. Appearance of brown flocculent precipitate indicates the presence of alkaloids.

Wagner's reagent: Iodine 1.27 grams and 2 grams of potassium iodide were dissolved in 5 millilitres distilled water and solution was further dissolved in water to make final volume 100 millilitres.

(C) Hager's test

Few millilitres of filtrate were taken in a test tube and one or two millilitres of Hager's reagent were added. A prominent yellow color precipitate indicates positive test. Hager's reagent: A saturated aqueous solution of picric acid was made.

(D) Dragendroff's test

To a few millilitres of filtrate, 1 or 2 millilitres of Dragendroff's reagent were added. A prominent yellow precipitate indicates positive test.

Dragendroff's reagent: It was prepared by mixing solution A (17 grams of Bismuth subnitrate + 200 grams of tartaric acid + 800 millilitres of distilled water) and solution B (160 grams of potassium iodide + 400 millilitres of distilled water) in 1:1 proportion (V/V).

2. Test for carbohydrates

Extracts (100 milligrams each) were dissolved individually in 5 millilitres of water and filtered. The filtrates were then subjected to following test.

(A) Fehling's test: About 1 millilitre of filtrate was taken in a test tube and added with 1 millilitre of Fehling A and 1millilitre of Fehling B solution and mixed well by shaking. The test tube was heated on water bath for 2 minutes. Appearance of red precipitate indicates positive test.

Fehling - A solution: Copper sulphate (34.66 grams) was dissolved in distilled water and volume made upto 500 millilitres.

Fehling - B solution: Potassiun sodium tartarate (173 grams) and sodium hydroxide (50 grams) were dissolved in water and volume made upto 500 millilitres.

(B) Benedict's test : About 0.5 millilitre of filtrate was taken in a test tube and 0.5 millilitre of Benedict's reagent was added. The mixture was heated over boiling water bath for 2 minutes. A characteristic colored precipitate indicates test as positive.

Benedict's reagent: Sodium citrate (173 grams) and sodium carbonate (100 grams) were dissolved in 800 millilitres of distilled water and boiled to make it clear. Copper sulphate (17.3 grams) dissolved in 100 millilitres distilled water was added to it.

3. Test for glycosides

The extract (50 milligrams) was dissolved in concentrated hydrochloric acid for 2 hrs on water bath, this hydrolysate was filtrated and filtrate was used for following test.

Legal's test

About 50 milligrams of extract was taken in a test tube and small amount of pyridine was added to it and mixed well. After that sodium nitroprusside followed by 10% sodium hydroxide was added. Development of pink color indicates positive test.

4. Test for saponins

The extract (50 milligrams) was taken in stoppered test tube and finally diluted upto 20 millilitres by adding distilled water. The tube was shaken for 15 minutes and observed for formation of foam. A two centimeter foam layer indicates positive test.

5. Test for proteins and amino acids

The extract (100 milligrams) was dissolved in 10 millilitres of distilled water and filtered through Whatman filter paper no. 1 and filtrate was again used for following test. (A) Biuret test (Rosenthaler, 1930) A few milligrams of residue were taken in water and 1 millilitre of 1% solution of sodium hydroxide was added followed by a drop of 1% solution of copper sulphate. Violet pink color development indicates positive test for proteins.

(B) Ninhydrin test

Two millilitre of filtrate was taken in test tube and few drops of ninhydrin solution were added. A characteristic purple color indicates positive test for presence of amino acids.

Ninhydrin solution: About 10 milligrams of ninhydrin dissolved in 200 millilitres of acetone

(C) Xanthoprotein test (Rosenthaler, 1930) A little residue was taken in 2 millilitres of

water and to it 0.5 millilitre concentrated nitric acid was added. Appearance of white or yellow precipitate indicates presence of proteins.

6. Test for phytosterols

Salkowski's test (Rosenthaler, 1930. A small amount of extract was taken in 2 millilitres of chloroform and sulphuric acid was added alongside of test tube and test tube was shaken. Red color development in the chloroform layer and greenish yellow fluorescence in the lower layer indicates presence of sterols.

7. Test for phenolic compounds

(A) Ferric chloride test

About 50 milligrams of extract were dissolved in 5 millilitres of distilled water and transferred to test tube and to this 5% neutral ferric chloride solution was added. Development of dark green color indicates presence of phenolic compounds.

(B) Lead acetate test

About 50 milligrams extract was dissolved in distilled water and 3 millilitres of 10% lead acetate solution were added. A bulky white precipitate indicates presence of phenolic compounds.

8. Test for flavanoids (Rosenthaler, 1930)

A small quantity of residue was dissolved in 5 millilitres of ethanol (95%) and treated with a few drops of concentrated hydrochloric acid and 0.5 gram of magnesium metal turnings. Development of either pink or red color indicates presence of flavonoids.

9. Test for resins (Rosenthaler, 1930)

The alcoholic extract was dissolved in alcohol. To this, a few drops of water were added. The appearance of turbidity was considered as a positive test.

RESULTS

The fresh procured leaves with four different solvents i.e. distilled water, ethanol, acetone and petroleum ether were extracted. The percent extractability of *Limonia acidissima* leaves in water, ethanol, acetone and petroleum ether solvents were calculated as 24%, 14%, 2% and 1% respectively. The percent extractability, colour and consistency of individual extract in details are given in **Table 1**. The detailed phytochemical investigation of plant extracts were done to detect the presence or absence of various classes of phytoconstituents present in the extract. The maximum phytochemicals were detected from an aqueous and ethanolic extract. The phytoconstituents like alkaloids, carbohydrates and glycosides, proteins and amino acids, saponins, phenols, resins, etc. were detected. The results obtained in phytochemical analysis are presented in **Table 2**.

DISCUSSION

The percentage of extractability obtained through this extraction process is in accordance with the results obtained in the previous work done by various researchers. The highest percentage of extractability was obtained through aqueous extract i.e. 24%. However, percentage of extractability in water is not found in reviewed literature. The percentage of extractability with

ethanol is recorded as 14% in present study which is almost double as compared to the extractability (7.89%) mentioned by **Panda *et al.,* 2013**. The extractability with petroleum ether has been mentioned as 1% by **Panda *et al*., 2013** which is similar to our results. However, **Baneerjee *et al*., 2011** mentioned the total yield of 1.12 grams of petroleum ether extract; acetone extract 2.34 grams and alcohol extract 2.21 grams from 25 grams of leaves, which comes to 4.48, 9.36 and 8.84% respectively. As compared to this in present study petroleum ether and acetone extracts were less and alcoholic extract was yielded more (14%).

The aqueous extract showed presence of alkaloids, carbohydrates, proteins and amino acids, phenolic compounds and resins. As mentioned earlier phytochemicals in aqueous extract have not been mentioned in the literature it could not be compared with the present study. Ethanolic extract revealed glycoside and saponins in addition to those phytochemicals present in aqueous extract. The ethanolic extract revealed presence of phytoconstituents, which is in mere agreement with previous study by **Panda *et al.,* 2013** who detected all the phytochemicals in addition to phytosterols and flavanoids. **Singh and Vidyasagar, 2015** revealed the absence of steroids in leaf ethanolic extract which is in agreement with the present study. When the phytochemicals of the ethanolic extract obtained in this study compared with phytochemicals obtained in a study conducted by **Thilagavathi *et al.,* 2015**, it is observed that all the phytochemicals matched, except presence of saponin and absence of flavonoid and saponins in two studies. The acetone extract revealed alkaloids, phenols and resins, which were not found to be mentioned in reviewed literature. The petroleum ether extract in the present study revealed presence of alkaloids and resins. The results of phytochemical analysis with leaf petroleum ether extract seems almost match with previous results stated by **Attarde *et al*., 2011**, except for presence of steroids and absence of alkaloids. It is important to note that in the present study all the solvent extracts detected presence of alkaloids and resins. Many authors revealed presence of flavonoids and terpenoids/steroids in leaves, but not detected in this study from any of the tested extract. This variation could be due to the method of estimation of flavanoids which was followed in present study and alkaline reagent test followed by **Attarde *et al*., 2011**. However, resins were not detected by any author whichwere found in this study. However,these variations in presence of phytochemicals may be due to geographical and climatic conditions or habitat of the plant.

Hence, it can be said that water and ethanol are able to extract out most of phytochemicals and can employed for therapeutic purposes being repository of many phytoconstituents.

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Table No. 1. The colour, consistency and percent extractability of aqueous, ethanolic, acetone and petroleum ether extract of leaves of plant *Limonia acidissima.*

Sr. No	Type of extract	Quantity used	Colour	Consistency	Percentage of extractability
1	Aqueous	100 gm	Brown or coffee colour	Semisolid and pasty	24
2	Ethanolic (EtOH)	100 gm	Dark olive green	Semisolid and pasty	14
3	Acetone (Me_2CO)	100 gm	Dark green	Semisolid	2
4	Petroleum ether	100 gm	Sea weed green	Semisolid	1

Table No. 2. Phytochemical analysis of aqueous, ethanolic, acetone and petroleum ether leaf extracts of plant *Limonia acidissima.*

Sr. No	Active Principle	Test employed	Aqueous extract	Ethanolic extract	Acetone extract	Per. ether extract
1.	Alkaloids	Mayer's test	Absent	Absent	Absent	Absent
		Wagner's test	Present	Present	Present	Present
		Hager's test	Absent	Absent	Present	Present
		Dragendroff's test	Present	Present	Absent	Absent
2.	Carbohydrates	Fehling's test	Absent	Absent	Absent	Absent
		Benedict's test	Present	Absent	Absent	Absent
3.	Glycosides	Legal's test	Absent	Present	Absent	Absent
4.	Saponins	Foam test	Absent	Present	Absent	Absent
5.	Proteins and amino acids	Biuret test	Present	Absent	Absent	Absent
		Ninhydrin test	Present	Absent	Absent	Absent
		Xanthoprotein test	Absent	Present	Absent	Absent
6.	Phytosterols	Salkowski's test	Absent	Absent	Absent	Absent
7.	Phenolic compounds	Lead acetate test	Present	Present	Present	Absent
		Ferric chloride test	Present	Present	Absent	Absent
8.	Flavonoids	Test for flavonoids	Absent	Absent	Absent	Absent
9.	Resins	Test for resins	Present	Present	Present	Present

ASSESSMENT OF THE BACTERIOLOGICAL QUALITY OF LOCALLY FERMENTED COW MILK (NONO) IN MAIDUGURI, NORTH EASTERN NIGERIA

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ABSTRACT

Contamination of locally fermented cow milk (Nono) by pathogenic bacteria constitute a major public health problem. This was because contaminated milk was a good medium that can support the growth and propagation of microbial pathogens. This study was designed to assess the bacteriological quality of locally fermented cow milk consumed in Maiduguri, northeastern Nigeria. Totally, forty (n = 40) samples of fresh, locally fermented cow milk (Nono) were collected and used in this study. Ten (10) milk samples each were collected from four randomly selected selling points (Monday Market, Custom Market, Kasuwan Shanu and Tashan Bama). The bacteriological quality of each milk sample were analysed using standard procedures for isolation, identification and enumeration of pathogenic bacteria. The mean total plate count ranged from $1.44x10^8$CFU/mL to $10.31x10^8$CFU/mL, while the mean total coliform count range from $0.57x10^8$*CFU/*mL to $11.17x10^8$*CFU/*mL. Statistically, there was a significant difference ($p<0.05$) in the mean total plate count and coliform count of milk samples collected from the four sampling points.Bacteriological culture and biochemical identification of all the isolates revealed *Escherichia coli*29(72.5%),*Klebsiella* specie3(7.5%),*Salmonella* specie 2(5%),*Staphylococcus aureus* 14(35%) and*Streptococcus* specie15(37.5%)respectively.All the results were above the acceptable limits (1.0 to 3.0×10^5 *CFU*/mL for total plate count and no coliform count per 100ml of milk sample)set by NAFDAC. Therefore the Nono products consumed in Maiduguri was not safe and wholesome for public consumption. Hence, the need to ensure the maintenance of standard hygienic protocols during the collection, storage, processing and marketing of locally fermented cow milk (Nono) in order to minimize microbial contamination.

Key words: fermented cow milk, Nono, Maiduguri, bacterial contamination.

INTRODUCTION

Preservation of milk by fermentation process is an age long tradition in Africa, Middle East, Asia, and Europe (Savadogo *et al.*, 2004). Locally fermented cow milk (Nono) forms part of a normal daily diets of most of the families in the northern part

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of Nigeria, especially among the nomadic Fulani family who owns more than 80% of the cattle population (Obi and Ikenebomeh, 2007). In Nigeria, cow milk is consumed immediately after collection, pasteurized or incorporated as an infant food supplement. Additionally, Nono also serves as an important source of income to many families in northern Nigeria. This is because of the general belief that locally fermented milk is more nutritious than the commercially processed yoghurt (Akabanda *et al.,* 2010; Egwaikhide *et al.,* 2014).

Despite its high nutritive content, cow milk can serve as an excellent culture medium for the growth and multiplication of microorganisms due to its complex nutrients and high water activity (Ashenafi and Beyene, 1994; Chye *et al.,* 2004). The commonly isolated bacterial flora found in fresh milk samples includes, *Streptococcus, Micrococcus*, *Lactobacillus, Lactococcus* and *Staphylococcus* spp. However, when milk is kept at a very low temperature before processing, the bacterial flora likely to be predominantly isolated from the sample are psychrotrophs. The isolation of coliform and other pathogenic bacteria from fresh milk samples usually occur as a result of contamination from processing equipment, hands of milkers or due to mastitis (Bonfoh *et al.*, 2003; Chye *et al.,* 2004). Fresh milk obtained from a healthy cow normally contains a low microbial activity, particularly with bacterial load of less than 10^3CFU/mL but, the bacterial load is likely to increase up to about 100 fold or more, if the milk is stored for some time at ambient temperature (Lingathurai and Vellathurai, 2010).

Contamination of milk by saprophytic bacteria not only reduces the nutritional quality of milk but also, constitute a significant health hazards to the humans beings (Godefay and Molla, 2000). Contamination of fresh milk sample can occur either at the stage of procurement, processing or distribution. It is pertinent therefore to identify the critical control points in order to ensure the production of safe and wholesome milk for human consumption. Important risk factors of milk contamination includes, the health status of the cow, the hygiene of the milking environment, hands of milkers, sources of water during processing , the hygiene of the utensils used to process the milk and lack of refrigeration facilities at both the farm and household (Mosu*et al.,* 2013). In addition, contamination of milk by microbes make it not only grossly unwholesome, but potentially harmful for human consumption. This is because, consumption of raw or unpasteurized milk has been reported to be associated with the occurrence of some important zoonotic and food-borne pathogens such as *Brucella abortus, Mycobacterium tuberculosis, Salmonella enterica, Yersinia* spp., *E. coli* O157 and Staphylococcal enterotoxins(Baylis, 2009).

In Nigeria, fresh milk obtained from cows was usually being processed via traditional method of fermentation by pouring the milk into a clean semi-dried container made from calabash (Egwaikhide *et al.,* 2014). The procedure was crude and unhygienic, hence serving as an important medium for the growth of pathogenic bacteria that could constitute significant public health problem (Emmanuel *et al.,* 2014). In addition, most cow milk sellers

were street peddlers and the milk was sold in an open environment by the road side; thus, potentially increasing the rate of contamination by bacterial pathogens. In some cases, left over milk was mixed with freshly prepared milk product without any special pretreatment to reduce the bacterial load (Okonkwo, 2011). To this end, this study was designed to assess the bacteriological quality of locally fermented cow milk in Maiduguri, Northeastern Nigeria.

MATERIALS AND METHODS

Samples collection

A total of forty (n = 40) freshly prepared fermented cow milk (Nono) samples were randomly purchased from four different milk selling points (10) each within Maiduguri Metropolis (Monday market, Custom market, Kasuwan shanu and Tashan Bama). Two hundred milliliters (200 mL) each of milk samples were collected in a sterile pre-labelled sample bottle, stored in ice and transported within 1-2hour to Veterinary Microbiology Laboratory, Faculty of Veterinary Medicine, University of Maiduguri for bacteriological analysis. A total volume of 25 mL of the milk sample was poured into a sterile plastic bag containing 225 mL of sterile distilled water and then homogenized with the aid of a stomacher.

Microbiological analysis

The milk samples were assessed for their bacteriological quality and as well as the occurrence of selected pathogenic bacteria. Enumeration of bacteria of interest using total plate count (*E. coli*, *Salmonella* spp and *Staphylococcus* spp) was carried out as per the guidelines of American Public Health Association(Vanderzant, 1992). Each sample was serially diluted using sterile distil water as described by (Obande et al., 2017) with little modification. Enumeration of the total numbers of *E. coli* and coliform bacteria in each milk sample was carried out using the three tube Most Probable Number (MPN) technique. Positive tube samples were cultured onto Eosin methylene blue agar (EMB) and then incubated at 37 ºC overnight. Colonies were confirmed based on colonial morphology, Gram staining for cellular morphology and biochemical test as described by (Cheesebrough, 2004; Egwaikhide *et al.,* 2014).

RESULTS AND DISCUSSION

The present study investigated the bacteriological quality of locally fermented cow milk (Nono) sold in Maiduguri, northeastern Nigeria. Cow milk is known to serve as a very good medium that supports the growth and multiplication of bacterial pathogens with undesirable consequences to human health. The presence of coliform in milk samples indicates gross contamination. Coliforms are considered as normal flora of the intestinal tract of human and animals, their presence in milk is indicative of fecal contamination. (El-Bakri *et al.,* 2009).

The result obtained showed that the milk samples collected in Tashan Bama had the highest mean Total plate count (TPC) ($10.31x10^8$CFU/mL) than those collected from Monday market ($1.44x10^8$CFU/mL) (Table 1). Additionally, there was a significant difference ($P<0.05$) between the mean TPC of milk samples collected from

Custom market, Kasuwan shanu market and those from Monday market and Tashan Bama. However, no significant difference ($p>0.05$) was observed between the mean TPC of milk samples collected from Custom market and Kasuwan shanu market. The mean TPC (of $5.5x10^8$CFU/mL) obtained in this study is above the accepted standard value 1.0 to *3.0 x 10^5 CFU*/mL for TPC set by National Food and Drug Administration and Control (NAFDAC) 2005. It was also higher than($3x10^3$CFU/mL - $25x10^3$CFU/mL)reported by(Egwaikhide *et al.,* 2014). This higher values could be due to one of the following reasons, the difference in the quality of the milk sample, the farm management practice, the method of collection, the health status of the cow, the geographical location and season, the method of processing, storage and distribution. This was because contamination of milk could occur through the hands of milkers, the use of unhygienic utensils and water during collection, storage and processing of the milk (Egwaikhide *et al.,* 2014). Furthermore, the result of this study also showed that milk samples collected from Tashan Bama had the highest mean total coliform count (TCC) ($11.17x10^8$CFU/mL) than those collected from Monday market ($0.57x10^8$CFU/mL) and the result was statistically significant at ($P<0.05$). However, there was no significant difference ($p>0.05$) between the mean TCC of milk samples collected from Monday market and Custom market. The mean TCC ($5.27x10^8$CFU/mL) observed in this study was not in line with standard set by NAFDAC that no *E. coli*or coliform shloud be isolated per 100mL of processed milk. This was higher than (13.9×10^6 CFU/mL) reported by (Lingathurai and Vellathurai, 2010) in the province of Madurai in Southern India. Coliforms bacteria were considered as normal flora of the intestinal tract of human and animals, where by majority of them do not pose danger to consumers but some pathogenic strains of *E. coli* could cause serious systemic condition. The presence of coliform in milk indicates direct fecal contamination (Okiki *et al.,* 2018).

In this study, the frequency of occurrence of the different types of bacterial contaminants isolated from all milk samples analyzed revealed *Escherichia coli* 29(72.5%), *Klebsiella* specie 3(7.5%), *Salmonella* specie 2(5%), *Staphylococcus aureus* 14(35%) and *Streptococcus* specie15(37.5%)respectively(Table 3; Figure 1). The presence of these bacteria in milk sample could be detrimental to human health. This was because some of these bacteria had been implicated in cases of milk-borne gastroenteritis (Lingathurai and Vellathurai, 2010). *Escherichia coli* was the most frequent bacteria isolated from all the milk samples analyzed. The level of contamination by *E. coli* reported in this study was higher than the standard set by NAFADAC as no *E. coli* should be isolated per 100mL of milk sample. However this contradict what reported by(Maduka *et al.,* 2013) where none of the commercial yoghurt analyesd were contaminated by *E. coli*. The presence of *E. coli* in milk sample wasusually accompanied by the presence of other bacteria like *Klebsiella* spp and *Staphylococcus aureus* which might be associated with milk handlers, fecal contamination or mastitis (Okonkwo, 2011). In this study, *Klebsiella* spp was also isolated from all the samples with low frequency

of occurrences 7.5%. The presence of this bacteria in milk sample was usually due to contamination by feces. This pathogen could cause detrimental infection in humans, most especially in people who consume Nono. Muhammad *et al.*, (2009) reported that approximately 60-80% of all *Klebsiella* species isolated from feces and clinical specimen were*klebsiella pneumoniae*. Furthermore, *Salmonella* species were also identified in milk samples collected from two sampling locations. This was lower than 9.8% and 23% reported by (Okonkwo *et al.,* 2012) and (Godwin and Emmanuel, 2013) respectively. These pathogens had been reported as one of the most important causes of nosocomial infection worldwide and were resistant to almost all classes of clinically relevant antibiotics (Patel *et al.,* 2008). Their presence in milk sample was worrisome, because *Salmonella*spp was one of the most important sources of food-borne hospitalization worldwide (Emmanuel *et al.,* 2014). Other bacteria isolated from milk samples in this study were*Staphylococcus aureus* and *Streptococcus* species having35% and 37.5% frequency of occurrence respectively. Their presence in milk could be associated with the health status of the cows. It was possible that these milk samples were obtained from mastitic cows. *Staphylococcus aureus* and *Streptococcus* species have been reported as the most predominant causes of bovine mastitis in cow (Workineh *et al.,* 2002). Consumption of milk contaminated with these pathogens exposed human to serious health risk through the transmission of pathogenic bacteria that were resistant to a wide compendium of antibiotics. Thus, increasing healthcare cost, prolonged hospital admission stay and in severe cases death.

CONCLUSION

The findings of this study showed that the bacterial count of locally fermented cow milk (Nono) retailed in Maiduguri is above the acceptable limit (1.0 to *3.0 x* 10^5 *CFU*/mL for total plate count and no coliform count per 100ml of milk sample) set by NAFDAC. Hence, rendering the milk unfit for human consumption. Nono samples containing high level of bacterial contamination could exposes the consumer to a lot of health hazards.

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Conflicting interest

The authors had read and approved the final draft of this manuscript and wishes to declare that there is no conflicting interest with regards to the publication of this manuscript.

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Table1: Mean total plate count (TPC) of locally fermented cow milk (Nono) samples collected from four locations in Maiduguri Metropolis

Locations	No. of samples	Mean x10^8/mL	95% Confidence interval
MMKT	10	1.44 [a]	0.36 - 2.52
CMKT	10	4.14 [b]	1.18 - 7.10
KMKT	10	6.11 [b]	3.69 - 8.53
TBM	10	10.31 [c]	9.03 - 11.59
TOTAL	40	5.50	3.57 -7.44

Values along the same column differently superscripted differs significantly (P<0.05); MMKT (Monday Market), CMKT (Custom Market), KMKT (Kasuwan Shanu Market), TBM (Tashan Bama)

Table 2: Mean total coliform count (TCC) of locally fermented cow milk (Nono) collected from four locations in Maiduguri metropolis

Locations	No. of samples	Mean x10^8CFU/mL	95% confidence interval
MMKT	10	0.57 [a]	0.36 - 2.52
CMKT	10	3.09 [a]	1.18 - 7.10
KMKT	10	6.25 [b]	3.69 - 8.53
TBM	10	11.17 [c]	9.03 - 11.59
TOTAL	40	5.27	3.57 - 7.44

Values along the same column differently superscripted differs significantly (P<0.05); MMKT (Monday Market), CMKT (Custom Market), KMKT (Kasuwan Shanu Market), TBM (Tashan Bama)

Table 3: Frequency distributionof occurrences of the different bacteria isolated from locally fermented cow milk (Nono) samples collected from four locations within Maiduguri metropolis

Locations	Sample size	*Escherichia coli*	*Klebsiella* spp	*Salmonella* spp	Staphylococcus spp	Streptococcus spp
MMKT	10	5	2	1	2	5
CMKT	10	7	0	0	5	2
KMKT	10	8	1	1	3	3
TBM	10	9	0	0	4	5
Total	40	29	3	2	14	15

MMKT (Monday Market), CMKT (Custom Market), KMKT (Kasuwan Shanu Market), TBM (Tashan Bama)

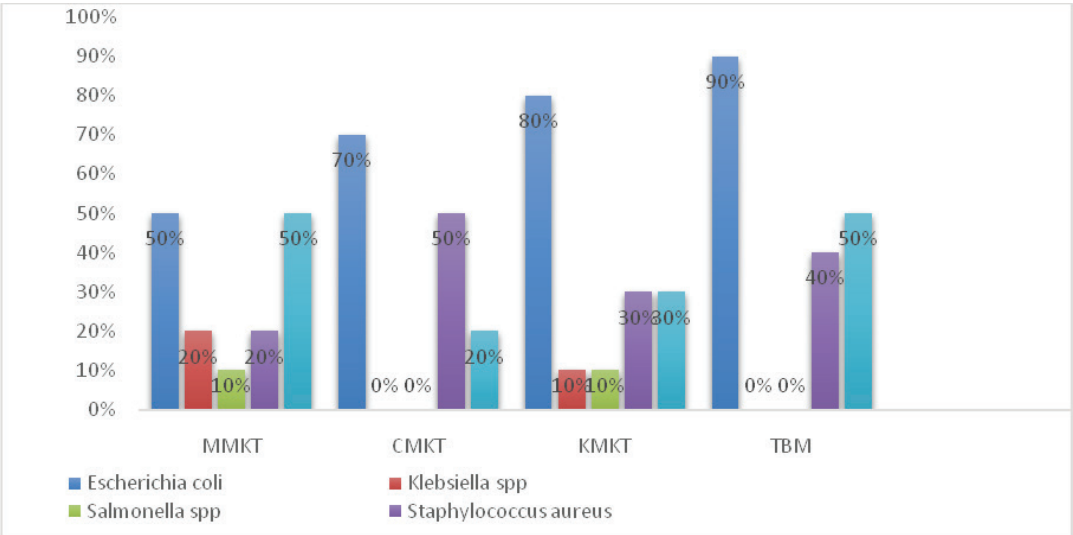

Figure: 1: Distribution of the overall mean (%) frequency of occurrences of the different bacteria isolated from fermented cow milk (Nono) samples.

EXVIVO STUDIES ON PHARMACODYNAMIC INTERACTION OF PHYTOCHEMICALS WITH ANTIBIOTICS AGAINST CLINICAL ISOLATES FROM MASTITIC MILK SAMPLES

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ABSTRACT

Drug interaction is the effect produced by combination of various drugs administered in a biological system which may produce beneficial or adverse effects.Due to continuous usage of antibiotics to bacterial infections, pathogenic bacteria developed resistance mechanisms which leads to reduced antibacterial activity of different antibiotics.Combination of antibiotics with phytochemicals became an alternative to enhance the efficacy of antibiotics. In this connection,in the present study, the effect of certain phytochemicals namely Quercitin,Morin, Ellagic acid,Chloragenic acid,Rutin and Naringenin in combination with antibiotics Amoxycillin and Ciprofloxacin were evaluated. *Staphylococcus aures*and *Escherichia coli* were used as test organisms. They were isolated from the mastitis milk samples and their presence was confirmed using Polymerse Chain Reaction .The antibacterial activity of the antibiotics and antibiotics in combination with phytochemicals weredetermined using broth dilution method and agar well diffusion assay. The MIC and zones of inhibition were used as indicators for drug interaction.

Key words: Drug interactions, Phytochemicals, *E.coli*, *Staphylococcus aureus*

INTRODUCTION

Mastitis is one of the common disease observed in livestock which causes production as well as economic loss to the farming community .Among the several causative agents of mastitis,the major bacterial species responsible are *Escherichia coli* and *Staphylococcus aureus*.These two bacteria are generally more prone for antibiotic resistance due to various reasons. Antibiotic resistance is the major drawback responsible for therapeutic failure in mastitis caused by the bacteria such as *E.coli* and *Staphylococcus aureus.* Even the selective antibiotics are available for the above bacteria commonly observed in mastitis cases the success of the treatment is not upto the mark till today.

Phytochemicals act synergistically with antibiotics , reduce the development of resistance to antibiotics and improve the efficacy of antibiotics (Savoia ;2012). Phytochemicals are the compounds present in plants also called as natural drugs which possess various pharmacological activities such as antineoplastic, antioxidant

, antibacterial, antiviral, anti-inflammatory and immunomodulatory activities.Natural compounds such as Oregano, Carvacrol and Thymol showed antibacterial activity by inhibiting the biofilm formation of *Staphylococcus aureus and Staphylococcus epidermidis* invitro.(Nostro; etal 2007). Similarly ,ascorbic acid influences the Ciprorofloxacin MIC against *Escherichia. coli* invitro (Srividya etal; 2017).As the natural compounds altering the antibacterial activity of phytochemicals,In the present study we determined the antibacterial activity of Amoxycillin and Ciprofloxacin against *Staphylococcus aureus*and *Escherichia coli* alone as well as in combination with selected phytochemicals Quercitin ,Morin, Ellagic acid ,Chloragenicacid, Rutin and Naringenin.

MATERIALS AND METHODS

Drugs and chemicals

Amoxycillin (Neovet), Ciprofloxacin, DMSO, Mueller Hilton broth (MHB), Mueller Hilton agar (MHA), Trypticsoya broth(TSB) were procured from Himedia. Querectin,Morin,Rutin, Ellagic acid and Chloragenic acid were procured from Sigma chemicals. All the phytochemicals were dissolved in DMSO for preparing the stock solutions of 10mg/ml.

Isolation of organim

Milk samples were collected from buffaloes suffering from clinical mastisis. The milk samples were inoculated into TSB and incubated at 37^0C for 18hr. After incubation the inoculum was streaked on Mac Conkey Agar, a differential media for *E.coli*. A single colony was then picked and streaked on Nutrient agar slant. The cultural characteristics of isolates were confirmed by streaking the pure culture on Eosin Methylene BlueAgar (EMB).The EMB agar showed greenish metallic sheen with black cantered colonies which indicates *E.coli*. The inoculums obtained from milk samples incubated in TSB at 37^0 C for 18 hours were streaked on Mannitol Salt Agar for the isolation of *S.aureus*. The *Staphylococcus aureus* showed yellow coloured colonies on MSA.

Identification of isolated bacteria by PCR

Isolation of bacterial DNA : The 18hr culture was inoculated in TSB and incubated at 37 °C for 18 hrs.After incubation 2ml of the bacterial culture was centrifuged at 5000rpm for 10 min. The pellet of bacterial cell mass was collected. The DNA template from bacterial cell mass was isolated by high salt method of DNA extraction (Aravindakshan et al; 1977) with suitable modifications.

The DNA template isolated was confirmed with primers Eco 2083 GCT TGA CAC TCA ACA TTG AG AND Eco 2745 GCA CTT ATC TCT TCC GCA TT (Riffon etal 2001).

The DNA template isolated was confirmed with primers Staur4 ACG GAG TTA CAA AG G ACG AC and Staur6 AGC TCA GCC TTA ACG AGT AC(Straub etal,1999)

Evaluation of antibacterial activity

Micro broth dilution method

A sterile 96 well flat bottomed plastic tissue culture plate with a lid was taken and filled 100 µl of MHB to all wells. 100 µl of the test compound was added to the first well and serially diluted. The 18hr bacterial culture was taken and adjusted to 0.5 Mc Farland standard.10µl of the bacterial culture was added uniformly to all wells and incubated at 37^0 C for 18- 24 hrs .The absorbance was taken at 660nm and the bacterial growth was confirmed by development of pink colour with the addition of *p*- Iodo NitroTetrazolium(INT). The minimum concentration of the test compound where it inhibits the bacterial growth is recorded and taken as minimum inhibitory concentration(MIC).(NCCLS 2000).

Agar well diffusion assay

The antibacterial activity of the selected phytochemicals was determined by agar well diffusion assay. 3.8 gm of MHA was dissolved in 100ml of distilled water and autoclaved at 121^0C for 15 min. It was allowed to cool and then poured in petriplates. After solidification the plates were inoculated with 18hr culture by sterile cotton swabs, then wells were created using a sterile cork borer and these were sealed properly with 1% agarose to avoid seepage of the test compound.10 µl of the test compound was added to the wells and plates were incubated at 37^0C for 12 – 24 hr. Areas of zone of inhibition was measured in millimetres using the formula πr^2

Statistical analysis

The differences of MIC values and zones of inhibition in agarose diffusion assays among various groups was tested using ANOVA as implemented in SPSS V17.

RESULTS

The mastitic milk samples were cultured in TSB. Genomic DNA was isolated from the culture to confirm the samples for the presence of *S. aureus* and *E. coli* using PCR technique (Figures 1 and 2). The positive samples of *S. aureus* and *E. coli* were further grown on specific media to isolate the specific organism. Thus isolated *S. aureus* and *E. coli* cultures were further used for microbroth dilution technique and ABST to understand the efficacy of antibiotic and natural compound synergistic activity. The pure antibiotic was used as control. The microbroth dilution technique indicated that Quercitin is the most efficient phytochemical against *S. aureus* among the tested natural compounds which is comparable with Amoxycillin (Table 1). Similarly, the pure form of Ciprofloxicin is most effective against *E. coli* and Morin is the most efficient phytochemical against *E. coli* among the natural compounds tested (Table 1).

The Amoxycillin and Quercitin combination was tested for ABST using *S. aureus* culture to understand the synergistic effect of the antbiotic and phytochemical antibiotic combination. When Quercetin alone was used, the zone of inhibition was very low. When Amoxycillin Quercetin combination was used, the zone of

inhibition was improved significantly and is comparable to the zone of inhibition of Amoxycillin alone used. (Table 2).

Based on the results of micro broth dilution technique, the combination of Ciporfloxacin and Morin was used to test the efficiency of antibiotic and phytochemical combination. In addition, the combination of Ciprofloxacin and Quercetin was also tested to understand the synergistic effect of the antibiotic and phytochemical combination. The combination of Ciprofloxacin and Morin was observed to be less efficient compared to Cirpofloxicin alone or Ciprofloxacin in combination with Quercetin (Table 3).

DISCUSSION

Antibiotics combined with natural compounds is of increasing interest in research to combat emergence of drug resistance.Application of the natural compounds for the treatment of ailments is an ancient practice. However, the natural compounds suffer from the disadvantage that their activity is weak in general and often non-specific (Srivastava et al., 2014). The modern antibiotics often suffer from development of resistance to microorganisms. Hence of late, a combination of phytochemicals and antimicrobials appears to be an effective method of dealing with antimicrobial resistance of antibiotics (Inui et al., 2007). Phytochemicals as such may not possess the significant antibacterial activity but application of these phytochemicals along with antimicrobial agents alters the pharmacological properties of antibiotic.(Z de sousa Silveria etal. ,2017)

In the present study we determined the antibacterial activity of phytochemicals, antibiotics alone as well as in combination by broth dilution method and Agar well diffusion assay. Quercetin showed lowest minimum inhibitory concentration compared to Amoxycillin against *S. aureus*, though the difference is not statistically significant. But agarose diffusion assay showed poor antibacterial activity of Quercetin alone against S. aureus. However, when Quercetin used in combination with Amoxycillin, due to synergistic effect, the combination produced strong antimicrobial activity that is comparable to the use of antibiotic alone at higher dose (Table2). The results are in agreement with previous studies indicating synergistic effect of Quercetin in combination with antibiotics (Kyaw et al 2012). Similarly combination of Quercetin and Morin with Ciprofloxacin also produced similar effect of usage of Ciprofloxacin alone even at lower doses (Table 3).

The present study suggests that usage of Quercetin in combination with antibiotics like Amoxycillin and Ciprofloxicin at lower doses can produce similar effect of the Antibiotic at higher dose. Such combination therapies may help in reducing resistance to antibiotics in mastitis therapy.

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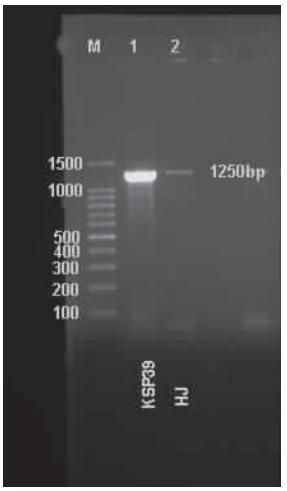

Figure 1 PCR amplification of *S. aureus* Lane M -100bp ladder,Lane 1- *S.aureus* isolate ,Lane2-*S.aureus* isolate

Figure 2. PCR amplification of *E. coli* Lane M -100bp ladder,Lane 1- *E.coli* isolate ,Lane2-*E.coli* isolate

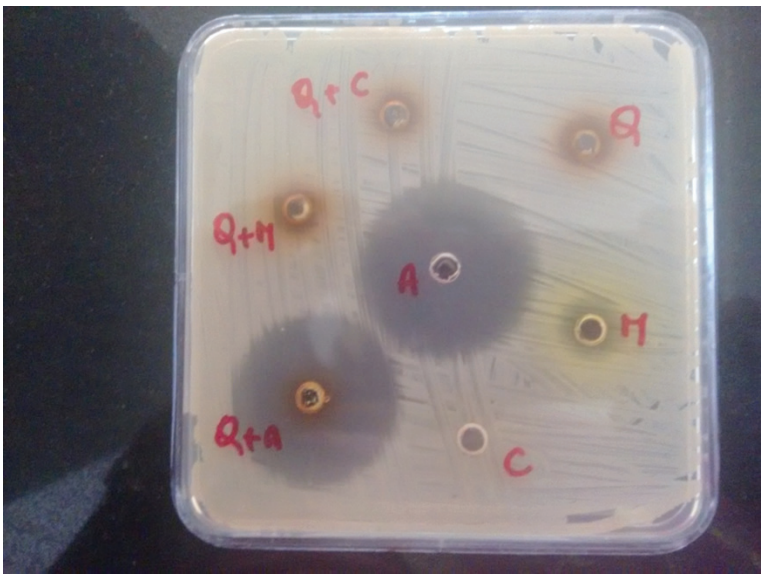

Figure 3. *Staphylococcus aureus* Agar well diffusion assay (C - Control, Q - Quercitin, M -Morin, A - Amoxycillin, Q+A - Quercitin+Amoxycillin, Q+M – Querctin + Morin, Quercitin+Chloragenic acid)

Table 1. Mean (+SEM) MIC values of the test compounds (mg/ml) aginst *S. aureus* and *E. coli*

S. No	Test compound	*S. aureus*	*E. coli*
1.	Quercitin	0.658±0.12^{a}	2.18±0.20^{bc}
2.	Morin	1.56±0.2^{ab}	0.81±0.13^{ab}
3.	Rutin	5±0.41^{d}	2.5±0^{bc}
4.	Naringenin	1.25± 0^{ab}	1.25±0^{ab}
5.	Ellagic acid	2.5±0^{bc}	2.5±0^{bc}
6.	Chloragenicacid	5±0^{c}	3.5±1.66^{c}
7.	Amoxycillin	1.14±0.1^{ab}	-
8.	Ciprfloxacin	-	0.0008±0.00015^{a}

Means with similar superscripts doesn't differ significantly (P>0.05)

Table 2. Agar well diffusion assay with *E. coli*

S. No.	Name of the test compound	Concentration	Area of zone of inhibition (mm^2)
1	Ciprofloxacin	10µl of 1mg/ml	1134.5
2	Ciprofloxacin+Morin	5µl of 5mg/ml + 5µl of 10mg/ml	962.5
3	Ciprofloxacin+Quercitin	5µl of 5mg/ml + 5µl of 10mg/ml	1134.5

Means with similar superscripts doesn't differ significantly (P>0.05)

Table 3. Agar well diffusion assay with *S. aureus*

S. No.	Name of the test compound	Concentration	Area of zone of inhibition(mm^2)
1	Amoxycillin	10µl of 5mg/ml	1213.34±68.86^{b}
2	Amoxycillin	5µl of 5mg/ml	962.50±0^{b}
3	Quercitin	10µl of 10mg/ml	86.82±11.67^{a}
4	Amoxycillin + Quercitin	5µl of 5mg/ml + 5µl of 10mg/ml	1174.91±20.17^{b}

Means with similar superscripts doesn't differ significantly (P>0.05)

EFFECT OF MULTI ENZYME SUPPLEMENTATION ON ILEAL DIGESTIBILITIES OF DRY MATTER, PROTEIN, ETHER EXTRACT, PHYTATE PHOSPHORUS AND NON-STARCH POLYSACCHARIDES IN BROILER DIET

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ABSTRACT

A biological trial was conducted to study the effect of multi enzyme supplementation on ileal digestibilities of dry matter (DM), crude protein (CP), ether extract (EE), phytate phosphorus (PP) and non-starch polysaccharides (NSPs) in terms of neutral detergent fibre (NDF), acid detergent fibre (ADF), hemicellulose and cellulose in broilers for a period of 0-6weeks. The broilers used in this trial were fed diets supplemented with enzyme at 0, 250, 500, 750 and 1000 g/ton of feed with a proportionate reduction in metabolizable energy (ME-1.25, 2.5, 3.75 and 5 %), crude protein (CP-0.75, 1.5, 2.25 and 3 %), methionine + cytine (0.5, 1, 1.5 and 2 %) and available phosphorus (2.2, 4.4, 6.6 and 8.8 %). The level of individual enzyme per gram of multi enzyme supplement was cellulase 146 IU, xylanase 241 IU, pectinase 98 IU, protease 74 IU, amylase 778 IU and phytase 33 IU. The ileal digestibility was conducted on 6th week of trial. The ileal DM digestibility (%) was significantly ($p<0.01$) increased in 750 g and 1000 g enzyme supplemented groups than other treatment groups. The increase in ileal DM digestibility was significantly ($p<0.01$) higher (6.2 %) in the group fed with 750 g enzyme supplementation than control. The digestibility of ileal crude protein (%) significantly ($p<0.01$) increased in 500, 750 and 1000 g enzyme supplemented groups than control. The increase in ileal protein digestibility was 12.20 % in 500 g/ton, 9.90 % in 750 g/ton and 10.12 % in 1000 g enzyme supplemented groups than control. However a non significant difference was observed in ileal EE digestibility in all treatment groups. The ileal phytate phosphorus digestibility significantly ($p<0.01$) increased in all the enzyme supplemented groups (33.69 % in 250 g/ton, 38.75 % in 500 g/ton, 39.53% in 750 g/ton and 42.11% in1000 g/ton) than control (31.71%). A highly significant ($P< 0.01$) increase in ileal neutral detergent fibre (NDF) and hemicellulose was observed in 500 to 1000 g enzyme supplemented groups than control. Similarly significant ($P<0.01$) increase in ileal acid detergent fibre (ADF) and cellulose digestabilities (%) were observed in all enzyme supplemented groups than control. It was concluded that the multi enzyme supplementation at minimum inclusion level of 500 g per ton of feed increased the ileal digestibilities of CP, PP, NDF, ADF, hemicelluloses and cellulose by 12.20 %, 18.17 %, 13.57 %, 55.63 % 12.77 % and 52.64 % respectively than control in broiler diet than the control diet in broilers.

Key words: Enzyme supplementation, ileal digestibility and nutrients

INTRODUCTION

The improvement of feed utilization is an important issue in poultry nutrition because of the need to reduce the environmental pollution from poultry and to decrease the production cost. Feedstuffs contain certain compounds like non starch polysaccharides (NSP's) and phytate phosphorus which either birds cannot digest or it may interfere with the bird's digestive system. Frequent reason for these indigestibility problems is that the birds are unable to produce the necessary enzymes to degrade these compounds (Khattak *et al*., 2006). In recent decades, much research has been performed in the study of chicken nutrition to investigate the use of exogenous enzymes to improve nutrient utilization (Leeson and Summon, 2005; Bharathidhasan *et al*., 2010; Truonga *et al*, 2017) and many commercial enzyme products are currently available for use in chicken nutrition. Further, the major challenge before the animal nutritionist is to minimize the feed cost without compromising the quality or to maintain the feed cost and improve the quality of the feed to exploit the genetic potential of the bird to the maximum extent. The shortage in availability of feed ingredients for poultry production compels to utilize the newer feed or alternative nutrient resources, which also contain higher levels of anti nutritive factors (ANFs) like non starch polysaccharides (NSPs) and phytate phosphorus. These ANFs interfere with the normal digestion process of birds there by reducing the availability of nutrients, due to lack of enzymes like cellulase and hemicellulase fractions, which are required for the utilization of the NSPs. Further, two thirds of the phosphorus in vegetable feed ingredients is present as phytate phosphorus, the utilization of which is limited due to lack of enzyme phytase in birds. The inclusion of enzyme preparations containing cellulase, hemicellulase and phytase was found to be helpful in enhancing the nutritive value of feedstuff with high NSPs (Friesen *et al*., 1992, Bharathidhasan *et al*., 2010) and phytate phosphorus (Simons and Vesteegh., 1990, Bharathidhasan *et al*., 2010). The mode of action of exogenous enzyme to improve the utilization of feed is well documented, which is directly related to digestion and hence the utilization can be studied using "Ileal Digestibility" experiment. Therefore this paper deliberates on the effect of multi enzyme supplementation on ieal digestibilities of nutrients in broilers on nutrient reduced diets.

MATERIAL AND METHODS

Five experimental broiler starter and finisher diets were formulated (BIS., 1992) containing commercial feed enzyme levels at 0, 250, 500, 750 and 1000 g per ton of feed with dose dependant reduction of metabolizable energy (ME-1.25, 2.5, 3.75 and 5 %), crude protein (CP-0.75, 1.5, 2.25 and 3 %), methionine+cytine (0.5, 1, 1.5 and 2 %) and available phosphorus (2.2, 4.4, 6.6 and 8.8 %). Each gram of feed enzyme contained the level of cellulase 146 IU, xylanase 241 IU, pectinase 98 IU, protease 74 IU, amylase 778 IU and phytase 33 IU. The ingredients and chemical composition (AOAC, 2007) of the formulated broiler diets are presented in **Table 1**. One hundred and sixty five Vencobb broiler straight

run chicks were wing banded, weighed individually and distributed randomly to five experimental diets with three replicates of eleven chicks each. The birds were housed in deep litter system from day one to six weeks and fed with weighed quantities of feed with *ad libitum* water following uniform standard managemental practices. The ileal digestibility was conducted by indirect method of digestibility using Titanium dioxide (TiO_2) as the external marker at sixth week of feeding trial and it was mixed at the rate of 5 g/kg of feed. Sixteen birds were selected randomly from each treatment group and they were provided experimental diet for five days before measurement of ileal digestibility in order to steady state of gastro intestinal conditions. After six days four birds per day per treatment were killed by decapitation, dissected immediately and the terminal ileal contents were squeezed out into a plastic container for four subsequent days. The terminal ileum was defined as equaling the length of the caeca plus 1 to 2cm ending 1cm from the ileo caecal junction (Danicke *et al*., 1997). Titanium dioxide was estimated according to the method of Short *et al* (1996) and the ileal digestibility was estimated for dry matter, crude protein, ether extract (EE), phytate phosphorus (Haugh and Lantzsh.,1993) and fibre fractionations (Goering and Vansoest., 1970) viz NDF, ADF, hemicellulose, cellulose. The data collected on various parameters were statistically analyzed as per the method of Snedecor and Cochran (1989).

RESULTS AND DISCUSSION

The mean ileal digestibility values of dry matter (DM), crude protein (CP), ether extract (EE) and phytate phosphorus are presented in **Table 2** and ileal digestibilities of NSPs viz. neutral detergent fibre (NDF), acid detergent fibre (ADF), hemicelluloses and cellulose are presented in **Table 3.**

The ileal DM digestibility (%) was significantly ($p<0.01$) increased in 750 g and 1000 g enzyme supplemented groups than other treatment groups. The increase in ileal DM digestibility was highest (6.2 %) in the group fed with 750 g enzyme supplemented group than control. Similarly, Steenfeldt and Pettersson (2001) also reported that the enzyme at 300 mg/kg improved ($p<0.02$) the ileal organic matter digestibility by 8.9 % compared with control. Langhout *et al.* (1997) observed that a significant increase in the digestibility of organic matter in endo-xylanase added to wheat and rye based broiler diets. The increase in dry matter digestibility might be due to the utilization of non starch polysaccharides, protein and phytate phosphorus while inclusion of feed enzymes in the diet.

The digestibility of ileal protein (%) was significantly ($p<0.01$) increased in 500, 750 and 1000 g enzyme supplemented groups than control. The increase in ileal protein digestibility was 12.20 % in 500 g/ton, 9.90 % in 750 g/ton and 10.12 % in 1000 g enzyme supplemented groups than control. A significant ($P<0.001$) improvement in ileal protein digestibility (Steenfeldt and Petersson, 2001) was found in earlier study in the chickens fed medium level of enzyme supplementation (200 mg/kg), increased the ileal protein digestibility by 12.4 % when compared to control. The enzyme supplementation was also increased ($p<0.05$) the ileal nitrogen digestibility

from 8 to 9.1 % in enzyme supplemented diet than control (Selle *et al*., 2009). Saki *et al*. (2005) also observed a significant effect (P<0.05) on ileal protein digestibility while in combination of different levels of enzyme and 2900 kcal/ kg ME in broiler chicken. The results of this study was also in par with earlier observations made by Baidoo *et al*. (1998) and Zanella *et al*. (1999) who noted that the influence of enzyme increased the ileal protein digestibility. Significant (p<0.01) increase in protein digestibility by 6.08 % was observed in the proximal ileum when 500 FTU/kg phytase included in the broilers diet than control (Truonga *et al*., 2017). Further, Friesen *et al* (1992) observed an increase in protein digestibility by 3 % in wheat based diet, 4 % in Bedford barley based diet, 7 % in rye based diet, 16 % in oat based diet and 20 % in scout barley based diet in the broiler chicken supplemented with crude cellulase enzyme. The increase in ileal protein digesibility is the result of the increased protein availability, which could be due to the release of trapped proteins by protease and phytase in the supplemented enzyme. Also the protease (74 IU/g) and phytase (33 IU/g) in the supplemented enzyme could have contributed to the increase in ileal protein digestibility.

There ileal EE digestibility showed only numerical increase in EE digestibility in all enzyme treated groups. Similarly Glamocic *et al*. (2011) and Selle *et al*. (2009) also reported that the exogenous enzyme had no effect on ileal EE digestibility in reduced ME diet. In contrary to the present study Steenfeldt and Petersson (2001) reported that the enzyme at 300 mg/kg increased (p<0.0007) the ileal fat digestibility by 17 % compared with control. Also, Allen *et al* (1997) reported that crude fat digestibility was significantly increased by 9.4 % when endoxylanase was added at a level of 100 mg/kg of animal fat based broiler diet. Multi enzyme supplementation numerically enhances the ileal digestibility of EE, but not significant in the present study might be due to the nutrients reduced diet used for broilers or the enzyme lipase was not included in the multi enzyme preparations.

The ileal phytate phosphorus digestibility was significantly (p<0.01) increased in all the enzyme supplemented groups (33.69 % in 250 g/ton, 38.75 % in 500 g/ton, 39.53% in 750 g/ton and 42.11% in1000 g/ton) than control (31.71%). The increase in ileal phytate phosphorus digestibility was from 5.88 % to 24.7 % in all the enzyme supplemented groups than control. Glamocic *et al*. (2011) also reported that the exogenous enzyme had improved the ileal total ash digestibility in reduced ME diet. Similarly Simons and Versteegh, (1990) reported that the addition of microbial phytase to the broilers fed with the diets containing 0.45% total phosphorus increased the availability of phosphorus from 49.8 % in the control to 56.5, 59.6, 59.6, 62.5 and 64.5% in 250, 500, 750, 1000 & 1500 Units per kg enzyme supplemented groups respectively. Edwards (1993) reported that the addition of phytase (600 U/Kg) decreased the fecal phytate phosphorus by 28 % than the control and the addition of 10 g/kg 1, 25 dihydroxy cholecalciferol further reduced the fecal phytate phosphorus content by 75 %. The result suggests that phytase enzyme present in the diet increased the utilization of phytate phosphorus. Also Carlos and

Edwards (1998) observed in laying hens that the addition 6000 U/kg of phytase to the corn soyabean diet containing 0.33 % total phosphorus enhanced the phosphorus retention by 83 %.

Table 2. Influence of multi enzyme supplementation on *ileal* digestibilities of dry matter, crude protein, ether extract and phytate phosphorus in broiler chicken.

Enzyme g/ton of feed	Dry matter (%)	Crude Protein (%)	Ether extract* (%)	Phytate phosphorus (%)
0	60.02 ± 0.25 [a]	72.57 ± 0.69[a]	69.58 ± 0.65	31.71 ± 0.20 [a]
250	61.15 ± 0.53 [a]	74.97 ± 0.16 [a]	69.78± 0.65	33.69 ± 0.19 [b]
500	61.94 ± 0.47 [a]	82.65 ± 0.88 [b]	71.16 ± 0.60	38.75 ± 0.28 [c]
750	63.99 ± 0.74 [b]	80.54 ± 0.91 [b]	71.21 ± 0.69	39.53 ± 0.21 [d]
1000	63.98 ± 0.47 [b]	80.74 ± 0.29 [b]	71.06 ± 0.56	42.11 ± 0.04[e]

Mean of four observations. *Non significant

Means bearing different superscripts in the same column differ significantly ($p<0.01$)

A highly significant ($p<0.01$) increase in ileal NDF digestibility (%) was observed in 500 g (40.74), 750 g (42.72), 1000 g (41.78)/ton of enzyme supplemented groups than control (35.21). The increase in ileal digestibility was by 13.57% in 500g/ ton, 17.58% in 750g/ton and 15.73 % in 1000g/ton over that of control. The NDF digestibility was also significantly ($p<0.05$) improved while supplementation exogenous enzymes in broiler diet (Glamocic *et al*., 2011). The finding was also in close agreement with Slominski and Campbell (1990), who observed an increase in the NSPs digestibility from 2.3 to 37 % when laying birds were fed with semi purified diet containing 40 % commercial canola meal (16-22 % NSPs) with 1 % enzyme.

Table 3. Effect of multi enzyme supplementation on ileal digestibilities of NDF, ADF, cellulose and hemicellulose in broiler chicken.

Enzyme g/ ton of feed	NDF (%)	ADF (%)	Hemi cellulose (%)	Cellulose (%)
0	35.21 ± 0.38 [a]	9.19 ± 0.10 [a]	39.82 ± 0.30 [a]	17.32 ± 0.41 [a]
250	36.02 ± 0.63 [a]	10.99 ± 0.25 [b]	40.28 ± 0.41 [a]	22.39 ± 0.47 [b]
500	40.74 ± 0.76 [b]	20.71 ± 0.22 [c]	45.65 ± 0.57 [b]	36.57 ± 0.62 [c]
750	42.72 ± 0.34 [b]	23.96 ± 0.26 [e]	47.35 ± 1.09 [b]	39.57 ± 0.62 [d]
1000	41.78 ± 0.61 [b]	22.37 ± 0.41 [d]	45.42 ± 0.75 [b]	39.32 ± 0.41 [d]

Mean of four observations. *Non significant

Means bearing different superscripts in the same column differ significantly ($p<0.01$)

The ileal ADF digestibility significantly ($p<0.01$) increased by 16.38 %, 55.63 %, 61.64 % and 58.92 % at 250, 500, 750 and 1000g/ton of enzyme supplemented groups respectively over that of control. The finding is similar to Manoj Sharma and Katoch (1993) who observed that the addition of Novozyme SP243 at 0, 15, 25 and 35 g/kg of layer diet increased ADF metabolizability significantly ($P<0.01$) in groups fed with 35g/kg of feed. Similarly Alloui *et al* (1994) who observed that a significant change in apparant digestibility of NDF, ADF when the enzyme was supplemented at 0.1% level to the diet containing 35% rapeseed meal compared with an unsupplemented enzyme group in broilers.

Ileal hemicellulose digestibility (%) was significantly ($p<0.01$) increased at 500 (45.65), 750 (47.35) and 1000g (45.42) of enzyme supplemented groups than control (39.82). The increase in hemi cellulose digestibility was by 12.77 %, 15.9 %, and 12.33 % in 500, 750 and 1000 g/ ton of enzyme supplemented group respectively than control. Glamocic *et al*. (2011) also observed that the ileal hemicelluloses digestibility was significantly ($p<0.05$) improved while supplementation exogenous enzymes in broiler diet. A highly significant ($P<0.01$) increase in the ileal cellulose digestibility was observed in 250, 500, 750 and 1000g of enzyme supplemented groups than control. The increase of cellulose digestibility was by 22.64, 52.64, 56.23 and 55.95 % in 250, 500, 750 and 1000 g of enzyme supplemented groups respectively over that of control. Similarly Slominsky and Campbell (1990) observed an increase in cellulose digestibility from 0.1 to 13% when the diet was supplemented with 1% enzyme in laying hens.

In commercial broiler production, the benefits of multi enzyme supplementation to NSPs rich diets are well documented (Bharathidhasan *et al*., 2010; Shim *et al*, 2017). In India, the maize and soybean meal are the major ingredients supplying the energy and protein in commercial broiler diets, however the other locally available feed ingredients like sunflower oil cake, rapeseed meal and de-oiled rice bran which contain higher level of NSPs and phytate phosphorus are also included in poultry ration to reduce the feed cost. Hence more poultry rations were formulated with locally available feed ingredients which contain higher level of NSPs and PP. The multi enzyme supplementation utilizes the unavailable portion of carbohydrate like NSPs and PP in poultry as documented early (Bharathidhasan *et al*, 2010; Shim *et al*., 2017) and also observed in the present study. The ileal digestibilities study of nutrients is an accurate measure to find out the efficacy of multi enzyme supplementation (Glamocic *et al*., 2011). As the multi enzyme supplemented in the present study increased the ileal digestibilities of CP, PP and NSPs viz.NDF, ADF, Hemicellulose and cellulose. The earlier studies also reported that the exogenous enzyme supplementation of broiler diets based on corn and soybeans improved the ileal digestibility of the nutrients in energy reduced diets (Glamocic *et al*., 2011) and total tract digestibility and performance (Bharathidhasan *et al*, 2009, 2010) like present study.

It was concluded that the multi enzyme supplementation at minimum inclusion

level of 500 g per ton of feed increased the ileal digestibilities of CP, PP, NDF, ADF, hemicelluloses and cellulose and by 12.20 %, 18.17 %, 13.57 %, 55.63 % 12.77 % and 52.64 % respectively than control in broiler diet which could be due to the presence of feed enzymes like cellulase, xylanase, pectinase, protease, amylase and phytase in the diet releases the trapped nutrients in bound form.

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Table-1: Ingredient and chemical composition of broiler starter and finisher diets

Ingredients (%)	Broiler starter (Enzyme inclusion (g/ton))					Broiler Finisher (Enzyme inclusion (g/ton))				
	0	**250**	**500**	**750**	**1000**	**0**	**250**	**500**	**750**	**1000**
Maize	44	39.5	35	28	23	47.50	43.00	36.00	31.20	25.00
Broken rice	2.2	7.0	8.0	14.0	18.0	4.80	6.00	9.20	14.40	15.80
Cumbu	2.8	2.0	4.0	5.0	6.0	3.50	5.80	9.00	8.00	12.70
Deoiled rice bran	1.1	2.1	5.0	5.6	6.3	2.60	4.50	5.80	6.65	7.20
Sunflower meal	0.5	0.8	0.5	0.5	0.5	0.50	0.50	0.75	0.70	0.70
Deoiled groundnut cake	8.0	8.0	6.8	6.8	6.4	3.80	3.75	4.65	6.65	10.50
Soya bean oil cake	37.8	37.0	37.1	36.5	36.2	32.70	31.80	30.00	27.85	23.60
Calcite	1.45	1.52	1.59	1.62	1.66	1.35	1.47	1.47	1.48	1.51
Dicalcium phosphate	1.65	1.58	1.52	1.49	1.44	1.75	1.69	1.63	1.57	1.47
Oil	0.5	0.5	0.5	0.5	0.5	1.50	1.50	1.50	1.50	1.50
DLMethionine (g/100 kg)	267.8	268.5	272.6	274.4	275.0	140.00	135.00	140.60	140.30	137.00

Nutrients	Broiler starter (Enzyme inclusion (g/ton))					Broiler Finisher (Enzyme inclusion (g/ton))				
	0	**250**	**500**	**750**	**1000**	**0**	**250**	**500**	**750**	**1000**
Dry matter	91.69	91.72	91.62	91.72	91.69	90.44	90.70	90.77	90.63	90.49
Crude protein (% reduction)	22.99 (0)	22.80 (0.75)	22.56 (1.5)	22.46 (2.25)	22.30 (3)	20.07 (0)	19.83 (0.75)	19.70 (1.5)	19.56 (2.25)	19.39 (3)
Crude fibre	4.86	4.83	4.99	5.17	5.33	4.83	4.95	5.20	5.44	5.24
Ether extract	2.95	2.71	2.65	2.41	2.47	3.93	3.85	3.79	3.70	3.46
Total ash	9.13	8.99	9.02	9.06	8.87	9.31	9.47	9.20	9.89	9.93
NFE *	60.07	60.67	60.78	60.90	61.03	61.86	61.90	62.11	61.41	61.98
Acid insoluble ash	1.96	1.99	1.94	1.92	1.96	2.06	1.96	2.12	2.08	2.29
Calcium	1.14	1.14	1.04	1.15	1.19	1.05	1.09	1.05	1.05	1.09
Phosphorus	0.67	0.69	0.68	0.68	0.67	0.67	0.68	0.69	0.68	0.67
AvailablePhosphorus* (% reduction)	0.45 (0)	0.44 (2.2)	0.43 (4.4)	0.42 (6.6)	0.41 (8.8)	0.45 (0)	0.44 (2.2)	0.43 (4.4)	0.42 (6.6)	0.4 (8.8)
Cystine +methionine* (% reduction)	0.90 (0)	0.90 (0.5)	0.89 (1)	0.89 (1.5)	0.88 (2)	0.70 (0)	0.69 (0.5)	0.69 (1.0)	0.69 (1.5)	0.69 (2)
ME(kcal/kg)* (% reduction)	2799 (0)	2767 (1.25)	2732 (2.5)	2695 (3.75)	2669 (5)	2904 (0)	2869 (1.25)	2829 (2.5)	2794 (3.75)	2758 (5)

1. Mineral mixture 1g per kg feed added and supplied calcium-6.4 g, phosphorus-1.2 mg manganese-55 mg, iodine-2 mg, zinc-52 g,copper-2 mg and iron-20 mg

2 .Vitamin AB_2D_3K 0.2g per kg feed added and supplied vitamin A-8250 IU, B2-5 mg, D3 1200 IU and vitamin-K-1 mg.

3. Coccidiostat 0.5g per kg feed added and supplied 125 mg of Di-nitro-ortho Toluamide -

4. Antibiotic (TM 100) 0.5 g added per kg of feed.

5. Feed Enzyme at the level of cellulase146 IU/g, xylanase 241 IU/g, Pectinase 98 IU/g, Protease 74 IU/g, Amylase 778

IU/g Phytase 33 IU/g

* Calculated values

Short Communications

SURGICAL MANAGEMENT OF LIPOSARCOMA IN PIGEON

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Liposarcomas are malignant tumours of lipocytes and lipoblasts and are uncommon in pet birds. This is in contrast to lipomas, which are benign neoplasms that are frequently reported in avian species (Reavill, 2001). Anatomic locations of liposarcomas described in birds include the regions surrounding the carpus, pelvic limbs and digits, neck, sternal subcutaneous tissues, uropygeal gland, abdominal cavity, and metastatic foci in the liver (Reavill, 2001) and Doster *et al*., 1987). Liposarcomas are usually pale yellow subcutaneous masses that are more firm, vascular, and infiltrative than lipomas (Reavill, 2001 and Petrak and Gilmore, 1982). Liposarcomas tend to act more aggressively than lipomas and surgical removal is recommended (Tully *et al*., 1994, Ritzman *et al*., 1996 and Berlin, 1988). This article reports the surgical excision of liposarcoma in a pigeon.

A three years old male pigeon weighing 400g (racing homer) was presented to Madras Veterinary College Teaching Hospital with the history of soft tissue growth on the keel region for the past six months and gradually increase in size was noticed. Physical examination revealed large tennis ball size soft tissue mass on the keel region (Fig. 1). Cytological examination revealed the presence of inflammatory cells with serosanguineous background. Radiological examination revealed presence of soft tissue mass on the keel bone with no bony involvement (Fig. 2).

Fig. 1. Large tennis ball size soft tissue on the keel region.

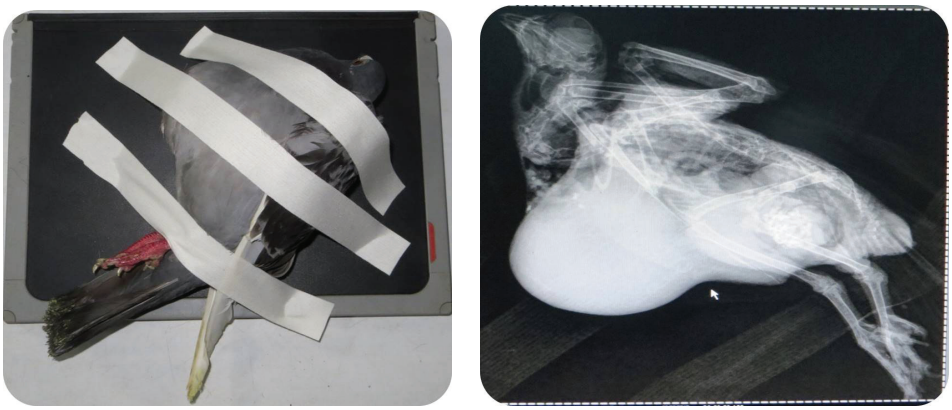

Fig. 2. Radiographic positioning of the bird and presence of soft tissue mass on the keel bone with no bony involvement

Surgical resection of tumour mass was performed under general anaesthesia. Food and water was restricted for 6h and 1-3h prior to surgery. The bird was pre-medicated with Atropine sulphate @ 0.04 to 0.1mg/kg b.wt IV (wing vein) and induced with ketamine @ 25mg/kg b.wt IM and diazepam @ 0.2 mg/kg b.wt IV. General anaesthesia was maintained with 2% Isoflurane in 100% oxygen through mask induction (Fig. 3). The surgical site was prepared aseptically (Fig. 4). The bird was positioned on dorsal recumbency. An elliptical incision was made over the tumour mass on the keel region (Fig. 5). The skin and fascia was removed. The tumour mass was resected from the keel bone (Fig. 6). No gross lesions of metastasis in other organs during the surgery. The subcutaneous tissues were closed in continuous suture pattern with PGA 2-0. The skin was sutured with 2-0 PGA by cross mattress pattern (Fig. 7). No intra-operative complications occurred during the surgery.

Fig. 3. The bird was prepared for surgery under general anaesthesia.

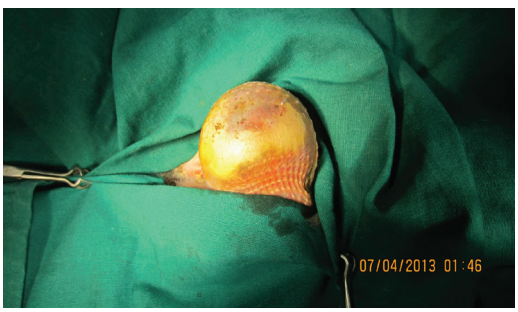

Fig. 4. Surgical site was prepared aseptically.

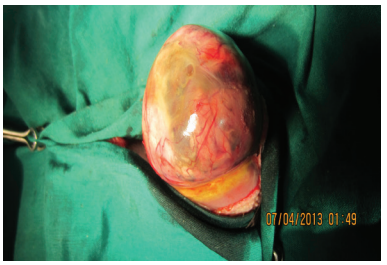

Fig. 5. Elliptical incision was made over the tumour mass on the keel region.

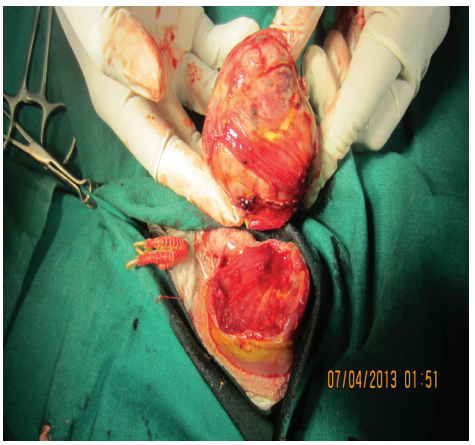

Fig. 6. Surgical resection of the tumour mass.

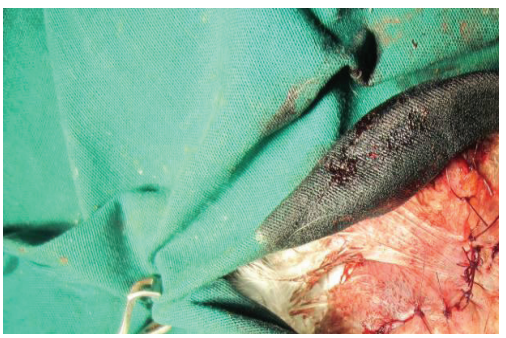

Fig. 7. The skin was closed by cross mattress pattern.

The excised tumour sample was collected in 10% formalin and sent for histopathological examination. Histopathological examination of the tumour mass revealed presence of polyhedral shape, vacuolated multiple fat cells and immature adipocytes. Histopathological examination confirmed liposarcoma (Fig. 8).

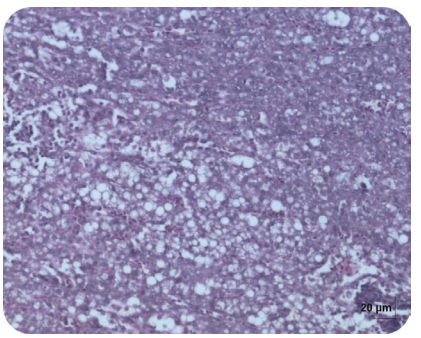

Fig. 8. Presence of polyhedral shape, vacuolated multiple fat cells and immature adipocytes. (Haematoxylin and Eosin stain, 100X).

Post-operatively, Tab Enrofloxacin @ 10mg/kg b.wt for five days and Tab Meloxicam @ 0.2mg/kg b.wt for three days were given orally. Owner was advised to give liquid food and water the day after surgery. The surgical wound was bandaged with mupirocin ointment every two days. No swelling and discharge was noticed. The bird recovered uneventfully within two weeks. There was no recurrence of the growth in a follow-up period of three months.

Lipomas are the most common tumour diagnosed in pet avian species (Campbell, 1986) and are identified most often in budgerigars, rose-breasted cockatoos, amazon parrots, and cockatiels (Reavill,

2004 and Latimer, 1994). Liposarcomas are a malignant variant of lipomas and are rarely diagnosed in psittacine species but have been described in budgerigars, cockatiels, macaws, monk parakeets, a pigeon and a green cheeked conure (Reavill, 2009, Tully *et al*., 1994 and Ritzman *et al*., 1996). Liposarcomas have a higher metastatic potential than lipomas and are locally invasive (Reavill, 2009). In many cases a definitive diagnosis of a lipomatous mass is easily made via the use of cytology. It is possible that one may not be able to cytologically differentiate between lipomas and liposarcomas, consequently a surgical biopsy may be necessary to achieve a definitive diagnosis (Reavill, 2004). In this case, cytological evaluation of the aspirated fluid from the tissue swelling was not helpful in finding a diagnosis of liposarcoma. Surgical excision has been reported to be the treatment of choice for treating lipomas in avian species (Harrison, 1986). In this case, the tumour mass was managed by surgical resection and confirmed by histopathological examination.

The signalment, physical examination and microscopic examinations are useful guidelines to aid in diagnosis. Results of fine-needle aspiration can be inconclusive, and complete surgical excision with histopathologic examination is recommended to establish a final diagnosis. In the above case, liposarcoma was successfully managed by surgical excision in a pigeon has been reported. No intra-operative complications occurred during the surgery. The recovery was good.

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A NOVEL SUPEROVULATORY PROTOCOL FOR ENHANCEMENT OF BOVINE *IN VIVO* EMBRYO YIELD

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Superovulation protocols are named according to the time from the first PGF2α treatment to the time of progesterone (P4) source removal, which occurs before the induction of ovulation to avoid the deleterious effect of high P4 concentration on embryo quality during the ovulation period. Therefore, when the PGF2 α is given on Day 6 AM and the P4 device is removed on Day 7 AM, the treatment is called "P-24" (i.e., a 24-h interval between PGF2α and P4 device removal). The purpose of this study was to evaluate embryo production and embryonic quality of cross bred cows, using two different protocols with different duration of progesterone exposure.

Six cross bred cows were gynaecologically examined by rectal palpation and ultrasonography before the start of the experiment, in order to determine their cyclicity and absence of diseases or abnormalities in their reproductive tract. The animals were divided into two groups; Group I: Treatments P12 (P12, n=3) and Group II: Treatments P24 (P24, n=3) in an experimental design.

The donor animals were selected with random stages of the estrous cycle (Day 0) and a progesterone device (TRIU-B® -Virbac Animal Health, São Paulo-SP) was inserted. The Superstimulatory treatment was initiated in D7 with the application of FSHp (Folltropin V ® - Bioniche Animal Health, Belleville, Ontario, Canada) in eight decreasing doses, administered at every 12 hours. Along with the fifth dose of FSHp, 500µg Cloprostenol (Pragma™, Intas Pharmaceuticals, India) was administered. The progesterone device was removed 12h (Group I) and 24 h (Group II) after the first injection of Cloprostenol. Twelve hours after the eighth dose of FSHp (D11) 25µg of Buserelin was administered (GnRH – Gynarich, Intas Pharmaceuticals, India) and the inseminations was done 12 and 24 hours later, using semen of bulls with known fertility. Embryos were collected in the forenoon of D18 of the schedule using nonsurgical method of embryo transfer. For the evaluation of the superovulatory responses ultrasound examinations were made for counting follicles and corpus luteum. An ultrasound scanner B Aloka SSD 500Vet (Aloka CO., LTD. - Tokyo,

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Japan) were used with a transrectal linear transducer 7.5 MHz: at D12 for counting superstimulated follicles (follicles ≥ 8 mm); at D15, for counting non-ovulated follicles (follicles > 8 mm) and at D20, for counting the number of corpus luteum before embryo collection. The embryos were collected by a non-surgical transcervical method with the aid of a Foley catheter. The uterine flush was performed with 1 liter of modified saline phosphate solution (ViGRO complete flush, Bioniche). The recovered embryos were evaluated using a stereoscopic microscope (Nikon SMZ645, Nikon ®, Tokyo, Japan) and classified according to the standards of the International Embryo Transfer Society (IETS, 1998), according to the stage of development (morulae, compact morulae, initial blastocyst, blastocyst, expanded blastocyst, hatching blastocyst and hatched blastocyst) and the quality (Grades 1, 2, 3, degenerated and nonfertilized ova). It was assumed that viable embryos would be those classified as grade 1, 2 and 3; freezable structures: grades 1 and 2; and unviable structures classified as degenerated or non-fertilized ova. All transferable embryos were vitrified using open pulled straw method for further use. The student's t test was used to compare the two different protocols with different duration of progesterone exposure.

The number of follicles after superovulation were 7.00 ± 0.58 and 17.33 ± 6.44 for P12 and P24 treatments, respectively suggested that the superstimulatory response could be greater with a possible adjustment of the doses used, as described by Nasser (2006) in *Bos indicus* (18.4 ± 3.4 and 23.0 ± 3.7, respectively) and Bó *et al*. (2002) in *Bos taurus* (18.6 ± 2.5). In general, *Bos taurus* and *Bos indicus* have different number of follicles (Baruselli *et al*., 2006) and *Bos indicus* recruit a larger number of follicles than *Bos taurus* (Carvalho *et al*., 2008) probably because they had higher plasma IGF-I and lower concentrations of FSH (Bo *et al., 2003*). No significant differences ($P > 0.05$) were found between treatments on the number of follicles after superovulation. This might be due to the different exposures to progesterone did not have significant effect on the number of follicles. The amount of non-ovulated follicles were different between P12 and P24 treatments(2.67 ± 0.67 vs 0.66 ± 0.33) possibly due to the injection of the same doses of FSH and to the ovulation induction with GnRH analogue, which has been performed at the same time in all experimental groups.

The number of corpus luteum at the time of embryo collection observed in this study in crossbred cows was 4.33 ± 1.2 and 16.33 ± 6.89 for P12 and P24 treatments, respectively. The donors of this breed have a great potential for embryo production with an adequacy of superovulation protocols. Carvalho *et al*. (2008) found that 22.4 ± 0.5 and 16.6 ± 3.4 corpus luteum for Nellore and Angus x Hereford, respectively. The differences found might indicate that future adjustments in superovulation protocols and selection of cross bred cows as donor animals may increase the superovulatory response.

The total number of structures collected was higher ($n=45$, $P > 0.05$) in cows superovulated with protocol P24 than in cows superovulated with the P12 ($n=11$) while the number of viable embryos was higher (41vs 6, $P > 0.05$) in P24 than

in the Control. There were no significant differences (P > 0.05) on the recovery rate and on the number of freezable and unviable structures between treatments. Silva *et al*. (2002) showed the positive effect of the higher plasma progesterone level on the quantity and quality of embryos in *Bos taurus*. The increase in progesterone due to the presence of the implant could be the reason for cows superovulated with P24 has a better embryo yield than cows superovulated with P12 treatment. Moreover, Carvalho *et al*. (2008) studied the effect of progesterone in a fixed-time artificial insemination (based on progesterone device) in *Bos taurus, Bos indicus* and *Bos taurus x Bos indicus* and observed that *Bos indicus* presented greater progesterone serum concentration than *Bos taurus* and crossbred animals. They argued that *Bos indicus* have lower speed to metabolize progesterone. Therefore, the more suitable protocol for *Bos indicus* would be P24, as this group keep higher progesterone levels for longer period, according to their metabolism, due to the anticipation in the removal of the progesterone device in these animals allowing that pulses of LH remain frequent and do not hinder the development of follicles (Baruselli *et al*., 2006). Thus, keeping the progesterone device longer, the influence of progesterone on the modulation of LH pulses was sufficient to prevent ovulation from occurring before the full maturation of follicle/oocyte (Carvalho *et al*., 2008). P24 presented better embryo yield than the P12 treatment, showing that these protocols are able to produce greater quantity of embryos with higher quality in crossbred cows. Embryo recovery rate were 65.56 ± 8.68 % and 89.77± 5.37% for P12 and P24 treatments, respectively (P > 0.05). The low recovery rate of the P12 treatment has resulted in fewer total and viable structures on the animals of this group. Furthermore, the smallest amount of viable structures presented by the P12 may be related to the concentration of progesterone supported only by corpus luteum formed in the pre-synchronization unlike the P24. Further trials will be conducted to facilitate the application of embryo transfer program to enhance the embryo yield.

The present study was conducted to evaluate embryo production and embryonic quality of cross bred cows, using two different protocols (P12 and P24) with different duration of progesterone exposure. P24 presented better results than the P12 treatment showing that these protocols are able to produce greater quantity of embryos with higher quality in crossbred cows.

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DETECTION OF SINGLE NUCLEOTIDE POLYMORPHISM OF A1/A2 VARIANTS OF BETA CASEIN GENE IN UMBALACHERY CATTLE BY TETRA ARMS PCR

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All over the world, people fulfill 13% of their protein requirements through milk and milk products (Cifelli *et al.,* 2015). Bovine milk contains two major protein groups namely caseins and whey proteins (Bell *et al*., 2006). Four types of casein protein present in bovine milk include alpha-S1, alpha-S2, beta and kappa casein of which, beta casein constitutes upto 35% of bovine milk protein. It can be present as either of the two major types - A1 and A2 owing to a single amino acid substitution in the 209 residues of the polypeptide chain (Farrell *et al.,* 2004). The A2 genetic variant contains proline at the 67th position of amino acid chain while A1 variant contains histidine. This amino acid substitution is the result of a natural genetic mutation leading to single nucleotide polymorphism of beta casein gene, wherein histidine is coded by CAT for A1 genotype and proline is coded by CCT for A2 genotype. Digestion of beta casein of bovine milk belonging to A1 variant favours the release of beta casomorphin (BCM), a bioactive peptide that possesses morphine-like opioid effects (Jinsmaa and Yoshikawa, 1999). Generation of beta-casomorphin is implicated as a major causative factor associated with A1 milk related health disorders like type 1 diabetes, cardiovascular disease, autism and neurological disorders in human (Wasilewska *et al*., 2011). However A2 milk not been linked with such health issues, as the A2 genetic variant that has a proline at that cleavage position does not favour the production of BCMs (Bell *et al.,* 2006). Animals may either be homozygous for the A1 or A2 allele or may be heterozygous with A1A2 alleles. Most often, exotic breeds are homozygous for the A1 allele whereas indigenous cattle like Gir, Tharparkar, Sahiwal and buffaloes are homozygous for the A2 allele (Mishra *et al*., 2009). Also, the frequency of beta casein genotypes varies with species, breeding programmes and geographical locations. Hence the present study was undertaken to highlight the genetic merit of a native breed of cattle - Umbalachery with reference to the A2 variant of the beta casein protein using tetra ARMS PCR.

Samples were collected from 45 Umbalachery cattle from Korkkai village of Thiruvarur district in Tamil Nadu. Five millilitres of blood were collected from the jugular vein of Umbalachery cows under aseptic conditions in sterile vacutainers coated with 2% EDTA and transported at 4˚C to the laboratory for DNA extraction.

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Bovine Genomic DNA was extracted from the whole blood using Phenol : Chloroform : Isoamyl alcohol method of Barker, 1998 without any modification. Beta casein (CSN2) variants for A1 and A2 variants were detected by adopting Tetra ARMS PCR method, using two sets of primers designed by Jaiswal *et al*. (2014) :

Outer forward: 5' CCGTTAATGAGAAATCCTTCAGYGAGCA 3'

Outer reverse: 5' TCTGGCTTTCAGTAAAGGGCTCAAACTGG 3'

Inner forward: 5' TAGTCTATCCCTTMCTGGGCCC**A**TTCA 3'

Inner reverse: 5' MGGGATGTTTGTGGGAGGCTSTCAG 3'

The 25 µl PCR mixture comprised of 12.5 µl of 2X PCR master mix, 100 ng of DNA, 3 µl of nuclease free water along with 1pM of each primer. The cycling parameters included an initial denaturation of 95˚C for 3 min, 35 cylces of denaturation at 95˚C for 30 sec, annealing in a touch down format from 59-54˚C for 30 sec, extension at 72˚C for 20 sec followed by a final extension of 72˚C for 5 min. The amplified PCR products were separated by electrophoresis on 2 % agarose gel in 1X TAE buffer (pH 8.3). After completion of electrophoresis, the gel was examined under UV illumination of a Gel documentation system.

A total of 45 numbers of Umbalachery cows was included in this study. Bovine genomic DNA was extracted from whole blood samples of Umbalachery cows following the Phenol : Chloroform : Isoamyl alcohol procedure. The concentration of the extracted genomic DNA estimated using the optical density at an absorbance of 260 nm was found to range from 62.88 ± 7.83 µg of DNA per millilitre. Yield of DNA was found to be comparable with the observations of Senthil (1995) wherein it ranged between 450 - 800 µg of DNA from 15 ml of blood samples of different breeds of Indian cattle. The observed optical density representing the absorbance ratio of the extracted genomic DNA at 260nm and 280nm with values of 1.89 ± 0.03 indicates the purity of the extracted genomic DNA and is appropriateness for genotyping procedures.

Genetic characterization of exon 7 of beta casein gene in Umbalachery cow was performed by Tetra ARMS PCR technique. Agarose gel electrophoresis pattern of the amplicons of Tetra ARMS PCR depicting beta casein variants of Umbalachery cows is presented in Figure 1. Three different sized amplicons were discernible. The larger amplicon with a fragment size of 256 bp served as positive control for amplication of partial beta casein gene. Depending upon the allelic variants, the other two amplified products obtained were either 199 bp or 98 bp. The amplicon of 199 bp was observed for the A1 allele and for A2 allele it was of 98 bp size.

Out of 45 animals studied, 38 cows were of A2A2 genotype (homozygous) and 7 cows were of A1A2 genotype (heterozygous). None of the Umbalachery cow in the present study belonged to A1A1 genotype. Similar pattern of presence of A2A2 and A1A2 genotypes was reported by Mishra *et al*. (2009) in Malnad Gidda

and Kherigarh breeds with absence of A1A1 genotype in fifteen *Bos indicus* cattle breeds that included Kangeyam, Kankrej, Gir, Sahiwal and Rathi. However, several authors have reported the occurrence of all three genotypes in *Bos taurus* cattle breeds like Holstein, Pinzgau and Simmental breeds (Miluchova *et al*., 2014).

The genotypic frequency of A2A2 genotype was found to be 0.844 in the present study while that of A1A2 genotype was 0.156. The gene frequencies of the A1 and A2 alleles was observed to be 0.078 and 0.922 respectively, indicating a higher frequency of A2 allele in Umbalachery cows. These observations are in agreement to the mean genotype frequency values of 0.974 for A2A2 and 0.026 for A1A2 genotype reported by Mishra *et al*. (2009) in fifteen selected *Bos indicus* breeds, while the mean A1 and A2 allele frequencies were recorded to be 0.013 and 0.987. In the same study by Mishra *et al*. (2009) and another report by Malarmathi *et al*. (2014), the frequency of A2 allele was almost 100% in Kangayem. In contrary, higher frequency of A1 variant was reported by Kaminiski *et al.* (2007) and Royo *et al*. (2014) in exotic breeds like Guernsey, Jersey, Holstein, Ayrshire, Danish Red and Asturiana de Los Valles. Thus it can be summarized that there is a preponderance or near fixation of A2 variant of beta casein gene in Umbalachery cattle similar to that of other zebu cattle and Indian river buffalo.

The A1 and A2 allelic variants of the beta casein gene were detected by Tetra ARMS PCR in Umbalachery cows. The gene frequency of the A2 allele was observed to be almost near unity with the result that the A2A2 genotype had a frequency of 0.84 and A1A2 of 0.156.

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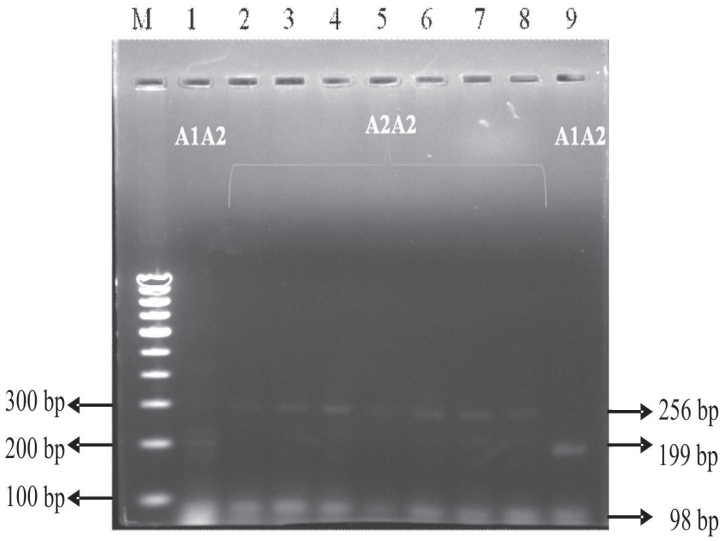
Fig 1: Agarose gel electrophoresis of the amplicons of tetra ARMS PCR depicting A1 and A2 variants of beta casein gene of Umbalachery cows



Lane M - 100bp DNA ladder
Lane 1 and 9 - Amplicons of A1A2 genotype
Lane 2 and 8 - Amplicons of A2A2 genotype

Table 1: Genotype and Gene frequencies of A1 and A2 variants of beta casein gene in Umbalachery cows

Genotypes	Variant distribution (N=45)	Genotype frequencies	Allele frequencies
A1A1	-	0	A1
A1A2	7	0.156	0.078
A2A2	38	0.844	A2
			0.922

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