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Clostridioides difficile infection: An emerging zoonotic disease

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Abstract

Clostridioides difficile is a spore-forming enteric pathogen of public health concern causing a toxin-mediated diarrhea in humans. In several countries, the bacterium has evolved as a hypervirulent, antibiotic-resistant pathogen with concerns for its nosomial and community-assocaited routes for disease transmission. Although the exact routes for community-associated infection have not been substantially elucidated, recent surveillance and genetic diversity analysis of community-borne isolates indicate for the potential spillover of the pathogen amongst the human, animal and environment interfaces. This review article highlights the importance of One Health approach for the control of *C. difficile* infection.

Keywords: *Clostridioides difficile*, zoonosis

Introduction

Clostridioides difficile is a significant human gut pathogen that causes a serious toxin-mediated enteritis in humans (Hookman and Barkin, 2009) . Annually, over 500,000 cases of *C. difficile* infections (CDI) are reported in the United States, which incur about $4.8 billion in healthcare and treatment costs (Lessa *et al.*, 2015). Being attributed as a nosocomial pathogen, *C. difficile* infection (CDI) has been increasingly observed among hospital in-patients undergoing long-term use of antibiotics, proton inhibitors and anti-inflammatory agents, which can lead to gut dysbiosis. Upon accidental ingestion of *C. difficile* spores, the dysbiotic intestinal milieu favorably initiates pathogenetic process of *C. difficile* to establish an intestinal infection (Bartlett, 1992; Kelly and LaMont, 1998; Dial *et al.*, 2006). However, during the last decade or two, evidence suggests that asymptomatic carriers and possibly other unknown sources outside the hospital settings may play a critical role in *C. difficile* transmission (Eyre *et al.*, 2013). For this reason, *C. difficile* has been suggested as a community-associated pathogen (Beaugerie *et al.*, 2003; Hensgens *et al.*,

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2012). More importantly, the increased reports of community-associated *C. difficile* infection (CA-CDI) is observed among young, healthy individuals, who were not previously exposed to antibiotics. In this review, we explain the current epidemiological scenario of CDI as a healthcare associated and community associated infectious disease, the potential of role of food, livestock, pet animals and the environment in contributing to the disease with emphasis to a One Health approach for the control of CDI (Fig. 1).

General taxonomical classification and epidemiological considerations

C. difficile is a gram-positive, anaerobic, spore-forming bacterium that can be found in humans, a wide range of animal species, and the environment (Weese, 2020). Previously, *Clostridioides difficile* was named as *Clostridium difficile* and was classified under the *Clostridium* sensu stricto group. However, a recent reclassification was made since *C. difficile* was shown to be phylogenetically distant from the rRNA clostridial cluster I and located in cluster XI. The cluster XI has been moved to the family *Peptostreptococcaceae*, and based on the phenotypic, chemotaxonomic and phylogenetic analysis, *C. difficile* was proposed to be renamed as *Clostridioides difficile* (Lawson *et al.*, 2016). Currently, both *Clostridium difficile* and *Clostridioides difficile* are validly used under the provisions of the Prokaryotic Code (Oren and Rupnik, 2018) .

During the late 1970's and 1980's, *C. difficile* was considered as a hospital-borne disease, which is responsible for causing pseudomembranous colitis and diarrhea in individuals who had undergone prolonged antibiotic therapy (Larson *et al.*, 1978; Lance George *et al.*, 1978). The importance of CDI was minimal during those times due to its reduced incidence rate and high recovery rate, since patients responded well to clindamycin, metronidazole or vancomycin administration. Although, recurrent CDI was documented in

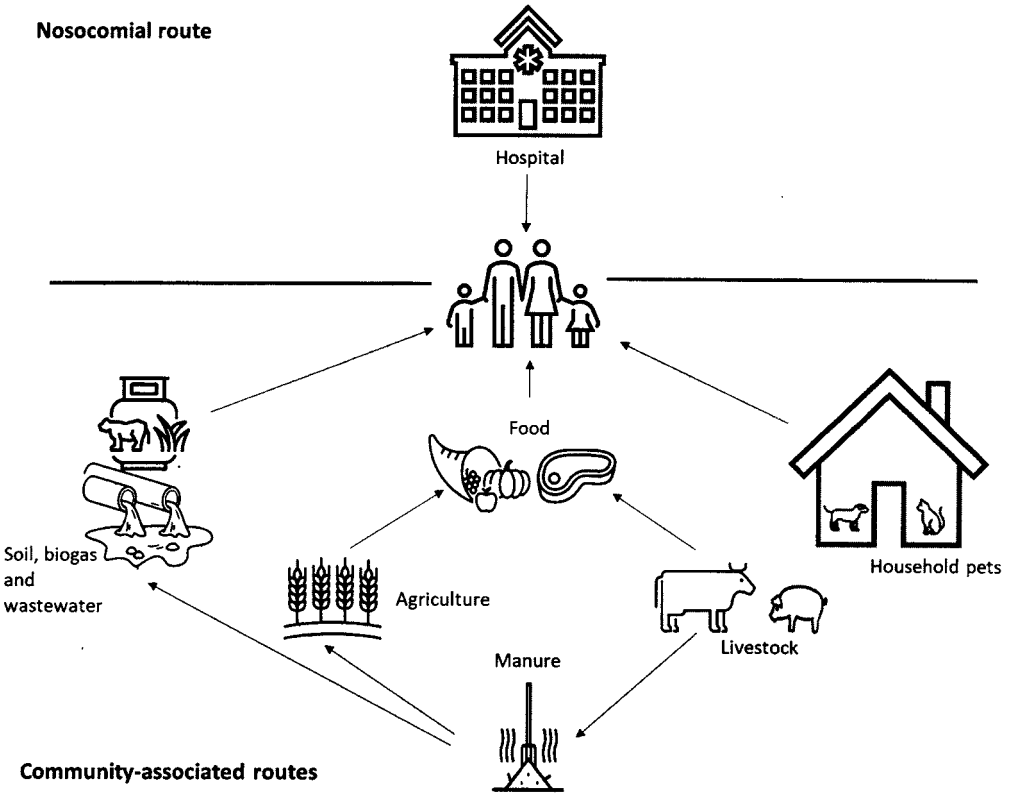

Fig. 1. Sources of C. *difficile* transmission routes to humans

the past, the condition was easily manageable with only infrequent incidences of the severe disease (Lance George *et al.*, 1978; George, 1988). However, in the early 2000's, a significant epidemiological shift occurred with the emergence of hypervirulent *C. difficile* evincing severe pathological implications and increased antibiotic resistance (He *et al.*, 2013). This fluoroquinolone-resistant hypervirulent strain known as pulsed-field gel electrophoresis type NAP1/restriction endonuclease analysis group BI/PCR ribotype (RT) 027 emerged in North America and later ensued to become a significant burden to the health-care systems worldwide (Spigaglia, 2016). The disease incidence was high mainly in high-income countries and is more commonly seen affecting the elderly, individuals with underlying medical illness, immunocompromised people and long-term hospital in patients requiring prolonged antibiotic therapy (Kim *et al.*, 2011). Although the incidence of CDI has not reduced over the past decade, the prevalence of hypervirulent ribotype 027 has declined in some countries (Turner *et al.*, 2019). Alongside this observation, several community-associated CDI (CA-CDI) cases have also been reported among low-risk individuals without any history of prior hospital admission for prolonged periods and in individuals not exposed to antibiotics (Beaugerie *et al.*, 2003; Lessa *et al.*, 2015; Turner *et al.*, 2019). Moreover, several investigators have documented the emergence of antibiotic resistance in *C. difficile*, especially against fluoroquinolones, clindamycin, erythromycin, metronidazole and vancomycin (Spigaglia, 2016). Consequently, the Centers for Disease Control and Prevention (CDC) in its report on emerging pathogens with antibiotic resistance, categorized *C. difficile* as one of the three urgent threats to public health (CDC, 2019). Early reports of *C. difficile* infection in India dates to the mid-1980s, wherein a prevalence study for *C. difficile* was performed in patients diagnosed with pseudomembranous and antibiotic-associated colitis in North India (Ayyagari *et al.*, 1986). Literature review based on *C. difficile* epidemiology in India reveals that there are only a few reports related to CDI incidence that have been recently published (Joshy *et al.*, 2009; Vishwanath *et al.*, 2013; Hussain *et al.*, 2016; Ghia *et al.*, 2021; Kannambath *et al.*, 2021). The prevalence rate for CDI reported in a tertiary care hospital in Kerala was in the range of 0.06-0.1%, whereas the prevalence rate ranged from 3.4-18.5% across the country (Ghia *et al.*, 2021). With the lack of information related to *C. difficile* prevalence in India, efforts need to be diverted for epidemiological and surveillance studies to monitor prevalence, risk factors and accuracy of *C. difficile* diagnosis for a better understanding of the disease burden in India (Ghia *et al.*, 2021).

In addition, recent CDI epidemiology findings over the past two decades indicate etiological implications for foodborne or zoonotic sources (Knight *et al.*, 2015). Besides food animal sources, *C. difficile* has also been isolated from soil, water, raw vegetables samples and milk (Jobstl *et al.*, 2010; Metcalf *et al.*, 2010; Janezic *et al.*, 2012; Hensgens *et al.*, 2012; Hoover and Rodriguez-Palacios, 2013; Kotila *et al.*, 2013). Although the exact routes of pathogen dissemination are not completely delineated, all reports suggest the likelihood of food and other environmental sources as plausible transmission routes of human CDI especially CA-CDI. Additionally, recent publications indicate that companion animals can act as a likely link for CA-CDI in humans (Hernandez *et al.*, 2020; Rodríguez-Pallares *et al.*, 2022). More information related to the epidemiological routes of healthcare associated and community associated CDI has been described in the subsequent sections.

Pathogenesis for nosocomial *C. difficile* infection

Humans develop CDI primarily by acquiring *C. difficile* spores through the feco-oral route (Hookman and Barkin, 2009). Patients suffering from CDI shed *C. difficile* spores which are resistant structures that can contaminate and survive in the hospital environment such as surfaces and equipments for months, and are extremely resistant to physical and chemical sanitizing agents (Kim *et al.*, 1981; Bettin *et al.*, 1994; Jabbar *et al.*, 2010; Siani *et al.*, 2011). Upon accidental ingestion of spores by susceptible patients, the spores transit through the gastric environment surviving the low pH conditions and eventually reaches the intestine. Susceptible individuals,

particularly those under prolonged antibiotic treatment will have a dysbiotic gut microflora. Under these circumstances, spores germinate to vegetative cells in the presence of primary bile salts present in the small intestine and the bacterium further establishes and colonizes in the distal gut (colon). In healthy individuals without antibiotic induced gut dysbiosis, distal gut microbiota readily transforms primary bile salts to secondary bile salts. A reduced availability of primary bile salts along with the suppressive action of secondary bile salts on *C. difficile* spore germination and vegetative growth helps to modulate colonization resistance against *C. difficile* by the host's healthy microbiota (Giel *et al.*, 2010; Theriot *et al.*, 2016). In susceptible individuals. upon reaching the colon, the vegetative *C. difficile* colonize and multiply in the intestinal crypts to produce major exotoxins, namely toxin A and B, which are critical virulence factors for CDI (Kuehne *et al.*, 2011). These exotoxins gain entry into the colonic epithelial cells and glycosylate the Rho and Rac GTPases by virtue of their glucosyltransferase domain (GTD). This reaction facilitates destabilization of critical cellular functions such as cytoskeletal disruption eventually leading to tight junction dissociation between colonic epithelial cells and the loss of epithelial integrity (Hunt and Ballard, 2013). As a result, there is an increased intestinal permeability that can favor translocation of bacteria from the gut lumen into deeper tissues (Naaber *et al.*, 1998). Mucosal epithelial damage results in the release of cytokines and chemokines such as IL-1β, IL-8, CXCL-1 and CXCL-2, which promote neutrophil recruitment and activation of resident dendritic cells and macrophage, favoring the release of additional proinflammatory cytokines, including IL-1β, IL-12 and IL-23. Subsequently, innate lymphoid cells are stimulated and release IL-22 and IFN-γ which upregulates macrophage and neutrophil phagocytic activity, production of antimicrobial peptides, reactive oxygen and nitrogen species (RNS and ROS). This process aids further to limit the translocation of other intestinal bacteria. Although inflammatory responses are essential for host survival after CDI, an overactivation of inflammatory responses proceeds to a condition called pseudomembranous colitis, which in advanced cases, can be detrimental to the host. *C. difficile* toxins in damaged epithelia further promote the release of cytokines such as IL-1, IL-8 and leukotriene-B, which further recruit more neutrophils to the affected region causing additional mucosal injury and focal micro-abscesses and pseudomembrane formation. In adverse conditions, an exaggerated immune response and release of systemically active cytokines, complicated by fluid loss from the resultant severe diarrhea may lead to systemic shock and death (Knight and Surawicz, 2013).

Potential routes for community associated C. difficile infection

Recently, there has been a considerable shift in the epidemiology of CDI (Knight *et al.*, 2015). Whole genome sequencing of isolates from symptomatic patients have shown that clinical *C. difficile* isolates were more diverse and the majority of the cases did not involve any sort of hospital contact. Moreover, reports of *C. difficile* transmission in households, between humans and animals (companion and farm animals), along with the isolation of toxigenic *C. difficile* from retail meat and vegetables suggest a more diverse source for human *C. difficile* acquisitions (Songer *et al.*, 2009; Rodriguez-Palacios *et al.*, 2014; Knetsch *et al.*, 2014; Loo *et al.*, 2016).

Livestock and companion animals as a source of C. difficile

The gastrointestinal tract of mammals (both humans and non-humans) are the preferred habitat for *C. difficile* and, young animals are more frequently colonized than fully-grown animals (Rodriguez *et al.*, 2016). *C. difficile* spores or toxin detection in piglets ranged between 1.4 and 96%, and up to 56% in calves less than three months of age (Rodriguez *et al.*, 2016). Decreased colonization in adult animals could be attributed to the colonization resistance offered by the adult gut microbiota. However, frequent use of antimicrobials, resulted in gut dysbiosis and reduced the colonization resistance resulting in food animals becoming a major source and amplification host for *C. difficile* (Moono *et al.*, 2016). Additionally, use of trehalose in swine production was also considered as a risk

factor for RT 027 and RT078 carriage in swine (Collins *et al.*, 2018; Turner *et al.*, 2019). The increasing number of *C. difficile* isolation from animals along with reports of RT078 isolation from humans with resistance to tetracycline (an antibiotic widely used in animals) indicates a possible transmission route of these isolates from food animals to humans (Dingle *et al.*, 2019). Foodborne transmission of *C. difficile* can also occur during slaughtering process where shedding of *C. difficile* occur with contamination of carcasses and meat as a result of gut spillage during evisceration or due to the accumulation of spores in the slaughter house (Weese *et al.*, 2011; Olivier Andreoletti, Dorte Lau Baggesen, Declan Bolton, Patrick Butaye *et al.*, 2013). *C. difficile* was isolated from the intestinal contents of up to 28% in pigs, 9.9% in beef cattle and 5% in broiler chickens. Moreover, genomic overlap of RT078 isolates causing human and porcine infections provides evidence for plausible transmission either directly from animals (foodborne transmission) or by an intermediate source (Indra *et al.*, 2009; Hopman *et al.*, 2011; Rodriguez-Palacios *et al.*, 2013; Moloney *et al.*, 2021). For example, feces of colonized or infected animal can act as transmission routes for human infection. Further, core genome analysis of RT078 from diverse sources in 22 countries across four continents revealed extensive clustering of human and animal strains indicating a potential bidirectional spread of *C. difficile* between farm animals and humans (Knetsch *et al.*, 2017). A compararive analysis of molecular characteristics of *C. difficile* isolates from humans and animals in North Eastern region of India also provided evidence that toxigenic isolates from cattle, pigs and poultry could potentially be a source of infection to humans or other animals (Hussain *et al.*, 2016).

However, lack of evidence directly linking food animal transmission and low prevalence of *C. difficile* in animal-derived foods resulted in the search of alternate sources for human infection. The detection of genetically identical and toxigenic *C. difficile* from companion animals, chiefly dog and cat suggests the potential role of household pet as a source for community associated CDI (Hernandez *et al.*, 2020). The close social interaction between companion animals and humans along with the use of similar antibiotics in both species provide a selective advantage and increases the incidental transmission of *C. difficile* in humans (Hernandez *et al.*, 2020). Although *C. difficile* could be normal members of intestinal flora in domestic animals, factors such as antibiotic treatments, changes in diet, poor intestinal motility, pancreatic dysfunction, presence of trypsin inhibitors and parasitic infections can alter the enteric environment of these hosts (Uzal *et al.*, 2016). This results in *C. difficile* overgrowth, which triggers sporulation and toxin secretion (Voth and Ballard, 2005; Uzal *et al.*, 2016). Toxigenic *C. difficile* was isolated from puppies at least once during the first 10 weeks of life (Perrin *et al.*, 1993). However, majority of the colonized dogs were asymptomatic with clinical features and pathogenesis strikingly different from humans. As an example, gut dysbiosis is not a significant feature in companion animals (Uzal *et al.*, 2016; Stone *et al.*, 2019). However, ribotypes shown to produce severe disease in humans such as RT 027, 078, 014/0 and 106 were isolated in dogs and cats and are often found to be antibiotic resistant. Recurrence of RT 106 in humans, a ribotype commonly found in dogs and cats was also reported (Silva *et al.*, 2015; Orden *et al.*, 2017; Rabold *et al.*, 2018). Recently, Rodriguez-Pallares *et al.* (2022) reported the first case confirming the transmission of C. difficile from a dog to a ten-month old female baby.

In India, although reports of *C. difficile* from animals are scanty, the bacterium was recovered from 31.5% of the dogs and 36.5% from pigs in North East India. Out of those positive samples, toxin genes were detected in 55.5% and 33.3% of dog and pig isolates respectively. In another report, toxigenic RT 012, 014 and 046 isolates were recovered from pet dog fecal samples in Assam, India (Hussain *et al.*, 2015; Das *et al.*, 2017). Further, study from Ludhiana, Punjab found *C. difficile* as the second important etiological agent causing diarrhea in canine patients (Sen *et al.*, 2019). Sequence based genotyping methods such as Multilocus sequence typing (MLST) and Multilocus Variable copy Numbers of Tandem Repeats Analysis (MLVA) have shown possible sequence types being shared between animals

and humans. For example, ST11 which involves the major human hypervirulent RT078 was commonly isolated in food animals (Griffiths *et al.*, 2010).

Food related sources of C. difficile

Considering the obligate anerobic nature of *C. difficile*, contamination of food products with endospores seems to be the possible transmission route of the pathogen in food sources. Although *C. difficle* spores have been detected only at low numbers in veal calf carcass, pork and beef, this might be significant as the spores are resistant to chilling, freezing and recommended cooking temperatures (71°C for over 2 hour) (Flock *et al.*, 2016). Presence of spores in the end products can occur as a result of initial contamination of the raw products, cross contamination of food or due to the production of spores during the processing steps (Rodriguez-Palacios and Lejeune, 2011). When present as spores in those food products, application of heat treatments might enhance the germination of spores and toxin production. However, on the other hand, *C. difficile* spores require a combination of bile salts (taurocholate, glycocholate, cholate and deoxycholate) for germination, and the lack of these ingredients in food matrices might not allow the spore to germinate. Increasing reports of *C. difficile* spore isolation from vacuum packaged and modified atmospheric packaged (MAP) foods also show that changes in food production can influence *C. difficile* prevalence in community settings (Broda *et al.*, 1996; Ghosh *et al.*, 2009; Bouttier *et al.*, 2010; Paredes-Sabja *et al.*, 2014; Atasoy and Gücükoğlu, 2017).

Although a majority of food related sources of *C. difficile* have focused on retail meats (beef, pork, and poultry), *C. difficile* has been isolated from a wide variety of foods, including vegetables (potato, lettuce, pea sprouts, ginger, carrot) and seafoods (clam, salmon, shrimp, mussels) around the world (Pirs *et al.*, 2008; Weese *et al.*, 2010, 2010; Gould and Limbago, 2010; Quesada-Gómez *et al.*, 2013; Rahimi *et al.*, 2014). In retail meats, *C. difficile* was isolated from 1.9 to 6.3% of the samples (von Abercron *et al.*, 2009; Jobstl *et al.*, 2010; Bouttier *et al.*, 2010; De Boer *et al.*, 2011; Rodriguez *et al.*, 2014). However, there has been a disparity in the prevalence of *C. difficile* in meats from North America and Europe with higher isolation of *C. difficile* from meats in United States and Canada than from Europe (Candel-Pérez *et al.*, 2019). Differences in sampling methods, size of operation, slaughtering practices and types of food examined could have resulted in such differences in prevalence. Prevalence of *C. difficile* isolated from fresh produce (fruits and vegetables) and minimally processed sea food ranged between 2.2 to 7.5% and 3.9 to 49%, respectively (Bakri *et al.*, 2009; Pasquale *et al.*, 2012; Eckert *et al.*, 2013; Troiano *et al.*, 2015). Higher prevalence of toxigenic *C. difficile* in mussels should not be underestimated, as these are eaten raw or partially cooked (Pasquale *et al.*, 2012). Contamination of prepared meals was also reported, and this could have originated from any of the ingredients or as a result of cross-contamination. *C. difficile* RT 017, 027 and 078 associated with community associated CDI were also reported to be isolated from food products (Goorhuis *et al.*, 2008; Weese *et al.*, 2010; Bauer *et al.*, 2011; Rodriguez *et al.*, 2014, 2015). However, no foodborne outbreaks have been reported until today and there have been no epidemiological studies that showed overlap between meat-associated and human infection strains (Turner *et al.*, 2019).

Environment related sources of C. difficile

Toxigenic *C. difficile* has been recovered from environmental sources such as wastewater, river sediments, soil and compost. Studies on *C. difficile* prevalence in European countries showed the greatest positivity rate of *C. difficile* (~100%) in wastewater treatment plant than from any other environment sources (Zidaric *et al.*, 2010; Kotila *et al.*, 2013; Steyer *et al.*, 2015; Moradigaravand *et al.*, 2018; Janezic *et al.*, 2020). Soil samples had unequal distribution of *C. difficile* depending on the soil type. As an example, soil from public environments such as parks, playgrounds, gardens and cultivated field had an overall prevalence rate of 4%, while soil collected from pastures, and paddocks in stables varied between 4 and 11% (Båverud *et al.*, 2003; Orden *et al.*, 2018). Similarly, depending on the substrates for the biogas plant (plant vs animal substrates), *C. difficile* positivity ranged between 4.5 – 58.8%, with

more positive samples when animal substrates were used in biogas plants (Fröschle *et al.*, 2015; Rodriguez Diaz *et al.*, 2018). Airborne spore transmission of *C. difficile* was also detected from within and around pig production farm with highest positivity recorded in samples collected in pens with neonatal pigs (Keessen *et al.*, 2011). However, the representation of environment as a true source of contamination or is due to the mere consequence of pathogen shedding by carrier or infected animals as a conduit for a specific environmental niche is not yet known. Core genome single nucleotide variant (cgSNV) analysis of *C. difficile* RT 104 of human and porcine isolates revealed interspecies transmission with 42% of human isolates overlapping with at least one animal isolate. However, these clones were recovered months and thousands of kilometers apart across different States of Australia, indicating indirect spread. This study suggests possible interconnected long-range zoonotic and/ or anthroponotic transmission that involves recycled waste products such as manure, biosolids and compost which could contaminate the crops resulting in widespread dissemination of *C. difficile*.

Current treatment strategies and novel therapeutic interventions for CDI

Although the prolonged use of antibiotics is known to predispose for CDI, antibiotics ironically remain to be the only approved treatment option for both human and veterinary CDI cases. Currently the recommended antibiotic therapy for CDI includes vancomycin, metronidazole and fidaxomicin. Other drugs not considered as primary choice of treatment include nitazoxanide, rifamixin, ramoplanin, tigecycline, and teicoplanin. The aforesaid antibiotics have been used for cases where severe and adverse effects have been observed with standard therapy, and where it is considered for salvage therapy in cases of fulminant CDI, multiple recurrences, and also where surgical interventions are impossible . Apart from generally used antibiotic agents, other novel antibiotics and adjunctive therapeutics have been developed, which are mostly undergoing human clinical trial evaluation. Some of the novel antibiotics under clinical trials include ACX-362E (synthetic purine targeting PolC type of polymerase of Gram positive bacteria), DS-2969b (binds the ATP binding site of DNA gyrase), Ridinilazole (inhibits bacterial DNA synthesis), Ramoplanin (a glycolipodepsipeptide antibiotic inhibiting transglycosylases required for peptidoglycan synthesis), DNV3681 (novel fluoroquinolone-oxazolidinone antibiotic), Cadazolid (oxazolidinone antibiotic), Surotomycin (daptomycin derived cyclic lipopeptide) and LFF571 (semisynthetic thiopeptide antibiotic blocking aminoacyl-tRNA delivery during translation) (Pellissery *et al.*, 2019).

Therapeutic adjunctive agents that can aid in restoring the normal gut flora is another treatment modality in C. difficile patients, which include fecal microbiota transplantation (FMT), standardized microbiota replacement therapeutics, probiotic bacteriotherapy and non-toxigenic *C. difficile*. These treatment approaches help to facilitate and modulate for the competitive exclusion of pathogenic *C. difficile* in the gut (Liubakka and Vaughn 2016; Khanna *et al.* 2017; Orenstein *et al.* 2015; Goldenberg *et al.* 2017; Mills *et al.* 2018; Júnior *et al.* 2019; Oliveira *et al.* 2016). Immunization strategies targeting toxin A and B are currently under different phases of clinical trials, however, the first US-FDA approved human monoclonal antibody therapy against toxin B is Bezlotoxumab (ZINPLAVA™) produced by Merck (Peng *et al.*, 2018). Some of the alternative and emerging strategies for CDI therapy include ebselen (glucosyltransferase domain binder), non-absorbable anionic polymers (Tolevamer), and phytochemicals and antimicrobial peptides (Mooyottu *et al.*, 2014, 2017; Furci *et al.*, 2015; Pellissery *et al.*, 2021).

Conclusion

In the past few years, there has been an increasing awareness related to the epidemiological shifts in *C. difficile* prevalence from a nosocomial etiology to a community associated pathogen. Particularly in the United States, community associated infections of *C. difficile* have increased totalling 61% of all CDI cases, suggesting a high possibility of additional sources that can cause CDI in non-hospitalized patients (Fu *et al.*, 2021). The identification

of genetic relatedness of *C. difficile* isolates derived from humans, animals and food sources plausibly suggests for potential zoonotic and anthroponotic networks for community associated CDI outbreaks (Lim *et al.*, 2020). Although, *C. difficile* control in humans relies on antibiotic stewardship and infection control in healthcare facilities, a critical evaluation from a One Health perspective focusing on potential human, animal and environmental routes for disease transmission will help to understand the epidemiological factors that play a role in *C. difficile* spread and in devising effective control measures.

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Epidemiological study of bacterial dermatitis in dogs of Wayanad district

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Abstract

A survey was undertaken to determine the epidemiology of bacterial dermatitis in dogs presented at the Teaching Veterinary Clinical Complex (TVCC), College of Veterinary and Animal sciences Pookode, from April 2017 to June 2018. Dogs of all age groups, breeds and both sexes with clinical signs of dermatitis were included in the study. Sterile swabs were used to collect samples aseptically from the dogs that showed clinical lesions of canine bacterial dermatitis. Samples were taken for culture and isolation of bacteria was done. There was no statistically significant difference among different age groups, however the highest occurrence was observed among dogs between 1-3 years (39.44 %) and out of 71 animals, 39 (54.93 %t) male dogs were positive for bacterial dermatitis, but no statistically significance among different sexes was observed. Among the various breeds studied, the highest occurrence was noticed in Labrador retrievers (23.94 per cent) when compared to other breeds but no statistically significance difference among different breeds was observed. Identification of bacterial isolates was done based on colony character, Gram's staining, oxidase test, catalase test, oxidative fermentative test and growth in specific media. A total of 71 bacterial isolates were obtained. Bacterial isolates obtained were Staphylococcus species (84.51 %), Streptococci (7.04%), Micrococci (5.63 %), and Pseudomonas species (2.82 %).

Keywords : *Epidemiology, bacterial dermatitis, Staphylococci, Streptococci, Micrococci, Pseudomonas*

Pyoderma is one of the most frequently encountered skin diseases presented to veterinary dermatologists and may account for 20 to 75 per cent of cases at Veterinary hospitals (Stegemann *et al*., 2007). In dogs, secondary bacterial infection may arise consequent to local trauma, scratching, poor grooming and resultant contamination, seborrhoea, parasitic infestation, hormonal factors, local irritants and allergies (Bajwa, 2016). Among different dermatological disorders the

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incidence of canine pyoderma was found to be 12.71 per cent and it was the third most frequently diagnosed skin disorder (Udayasree and Usha,2005). Staphylococcal pyoderma can also cause secondary complication with fungal infection and Malasseziosis (Daniel *et al.,* 2021).

The purpose of this study was to understand the influence of age, sex and breed on the occurrence of canine pyoderma and to identify organisms that caused bacterial dermatitis in dogs in Wayanad district.

Materials and methods

Samples

The present study was carried out at the Teaching Veterinary Clinical Complex (TVCC), College of Veterinary and Animal sciences Pookode, from April 2017 to June 2018. Dogs with clinical signs suggestive of bacterial dermatitis were included in the study. On presentation of the animal to the University Hospital, information on history, age, sex, breed, clinical signs *etc.,* were recorded. Before collecting samples, hair around the lesion was clipped and the area was shaved, swabbed with 70 per cent ethanol and air dried. Samples were collected using sterile cotton swabs kept in a screw capped tube. In the case of pustules, these were first swabbed and then opened using a sterile scalp or needle and touch swabs from the exudates were collected. In the case of crusty lesions, the crust was lifted and touch swabs were collected from beneath the crust. Samples collected were brought to the laboratory as early as possible for culture.

Isolation of bacterial organisms

Isolation of bacteria was attempted from pus by direct streaking on to Brain Heart Infusion agar (BHIA) followed by incubation of the plates at 37 ˚C for 24 hours. Plates were examined after 24 to 48 hours. Isolated colonies were selected and a representative sample was streaked on to BHIA slants for further identification. The isolates were stained by Gram's Method and depending on the preliminary characters, selective media were used. The isolates were identified based on morphology, cultural characteristics and biochemical tests as per Barrow and Feltham (1993) and Quinn *et al*. (2013).

Statistical analysis was done using SPSS version 24. Variables with $p<0.05$ were considered as statistically significant.

Results and discussion

Clinical signs

Dogs presented had generalised lesions, primary lesions and secondary lesions. Primary lesions included papules, pustules, nodules, erythema and alopecia. Secondary lesions were scales, crust, epidermal collarette, hyperpigmentation and pruritis. Similar lesions were observed in a study by Borio *et al.* (2015).

Age wise occurrence

The analysis of data on the occurrence of bacterial dermatitis revealed that this condition was observed in 28 dogs (39.44 per cent) between the age group 1-3 years followed by 18 dogs (25.34 per cent) below 1 year and the least occurrence of this condition was in dogs above 6 years (12.67 per cent) (Table 1). There was no statistically significant difference among the different age groups. The results were in close accordance with the observations of Shyma and Vijayakumar (2012).

Sex wise occurrence

Out of the 71 animals infected, 39 animals were male and 32 animals were female (Table 2). Though male dogs were affected to a greater extent, when compared to female dogs this difference was not statistically significant. The results were in concordance with those reported by Khurana *et al*. (2016).

Breed wise occurrence

The highest occurrence of bacterial dermatitis was noticed among Labrador Retrievers (23.94 per cent) followed by the German Shepherd (16.91 per cent), Pug (12.67 per cent), Rottweiler (11.27 per cent), Dachshund (8.44), Doberman (7.04 per cent), non-descript dogs (5.63 per cent), the Spitz

Table 1. Age wise occurrence of bacterial dermatitis in dogs

Sl. No.	Age Group	Number of infected dogs	Per cent (%)
1	Less than 1 year	18	25.35^{ns}
2	1-3 years	28	39.44^{ns}
3	3-6 years	16	22.54^{ns}
4	Above 6 years	9	12.67^{ns}
Total		71	100

ns – non- significant P<0.05

Table 2. Sex wise occurrence of bacterial dermatitis in dogs

Sl. No.	Sex	Number of infected dogs	Per cent (%)
1	Female	32	45.07^{ns}
2	Male	39	54.93^{ns}
Total		71	100

ns – non-significant P<0.05

Table 3. Breed wise occurrence of bacterial dermatitis in dogs

Sl. No.	Breed	Number of infected dogs	Per cent (%)
1	Boxer	1	1.41^{ns}
2	Dachshund	6	8.44^{ns}
3	Doberman	5	7.04^{ns}
4	Dalmatian	2	2.82^{ns}
5	German Shepherd	12	16.91^{ns}
6	Great Dane	2	2.82^{ns}
7	Labrador Retriever	17	23.94^{ns}
8	Non-descript	4	5.63^{ns}
9	Pug	9	12.67^{ns}
10	Pitbull	1	1.41^{ns}
11	Rottweiler	8	11.27^{ns}
12	Spitz	3	4.23^{ns}
13	Siberian Husky	1	1.41^{ns}
Total		71	100

ns – non-significant P<0.05

(4.23 per cent), Great Dane, Dalmatian (2.82 per cent) and the Siberian Husky, Boxer and Pitbull (1.41 per cent) (Table 3). There was no statistically significant difference among different breeds. The findings agreed closely with those made by Reddy *et al*. (2010).

Isolation and identification

The bacterial isolates obtained from clinical cases of dermatitis revealed that in 84.51 per cent samples *Staphylococcus* spp. was obtained while in 7.04 per cent, 5.63 per cent and 2.82 per cent of samples, *Streptococci*, *Micrococci* and *Pseudomonas* spp. (Table 4) were observed. The results obtained were similar to the findings of Shah *et al.* (2017).

Based on Gram's staining, catalase and oxidase test, 60 isolates were positive for *Staphylococcus* spp. followed by *Streptococci* (5 isolates), *Micrococci* (4 isolates) and *Pseudomonas* spp. (2 isolates) (Table 4). *Staphylococci* were grown in mannitol salt agar for confirmation. *Pseudomonas species* gave greenish yellow coloured colonies and Micrococci were observed as dark yellow coloured colonies in nutrient agar. The biochemical test was carried out as per Quinn *et al*. (2013) (Table 5).

Table 4. Number of bacterial organisms isolated from dogs skin

Sl. No.	Organisms Isolated	No. of isolates	Per cent (%)
1	*Staphylococcus* spp.	60	84.51
2	*Streptococci*	5	7.04
3	*Micrococci*	4	5.63
4	*Pseudomonas* spp.	2	2.82
Total		71	100

Table 5. Differentiation of bacterial isolates obtained

Sl. No.	Organisms	Gram's staining	Catalase	Oxidase	O/F test
1	*Staphylococcus* spp.	+ve cocci in clusters	+ve	-ve	F
2	*Streptococci*	+ve cocci in chains	-ve	-ve	F
3	*Micrococci*	+ve cocci in packets of four	+ve	+ve	O
4	*Pseudomonas* spp.	-ve rods	+ve	+ve	O

Conclusion

The present study revealed that age, sex, and breed of the dog had no statistical influence on the occurrence of canine pyoderma. However, among different breeds, the highest occurrence was observed among the Labrador Retrievers. Among the different age groups, dogs of three years of age were the most affected. Among the different bacterial organisms isolated, *Staphylococcus* spp was the predominant etiological agent.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Alkaline phosphatase in seminal plasma of sperm-rich fraction of semen in fertile and subfertile dogs#

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Abstract

Twenty dogs presented for breeding soundness evaluation were selected for the present study. Based on previous six month breeding history and semen quality, these animals were grouped retrospectively into fertile (n=6) and subfertile (n=14) groups. Three semen collections were made from each of these dogs, two weeks apart. Semen evaluation parameters like volume, sperm concentration, progressive motility, viability, morphologically normal sperms, total sperm output and alkaline phosphatase (ALP) level in the sperm-rich fraction were compared between the two groups. The mean values for progressive motility, sperm concentration, total sperm output and viability were 84.17 ± 1.54 per cent, 418.33 ± 36.64 million/mL, 645.11±98.43 million and 84.92 ± 1.56 per cent, respectively, in the fertile group, while the values for these parameters were 45.71 ± 3.47 per cent, 112.07 ± 24.36 million/mL, 190.24±32.45 million and 49.54 ± 2.35 per cent, respectively in the subfertile group. The values were significantly higher in fertile group when compared to subfertile group. The mean ALP (IU/L) was recorded as 168121.67 ± 22443.25 and 49605.86 ± 12669.68 IU/L, respectively in fertile and subfertile dogs. On statistical analysis, a highly significant difference ($p<0.01$) was noticed between the groups. Though the concentration of ALP was more than 40,000 IU/L (below which is the proposed cut off value for incomplete ejaculation) in all the dogs under the present study, it was significantly higher in fertile dogs, suggesting a possible relation to fertility.

Keywords: *ALP, fertile, subfertile, dog*

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Alkaline phosphatases (ALP) are membrane-bound enzymes attached to cells by phosphatidyl inositol glycan anchors (Low and Saltiel, 1988). ALP catalyze the hydrolysis of monophosphate esters at an alkaline pH. The exact role of this enzyme in semen is not known, however, desphosphorylation reactions may be essential for normal male reproductive physiology (Bell and Lake, 1962). In dogs and rabbits, ALP is primarily secreted by epididymis (Muller, 1983; Frenette *et al.,* 1986; Buonaguidi *et al.,*1991) whereas in humans, ALP is secreted by the testis and prostate (Kavanagh and Bardsley, 1979; Lewis-Jones *et al.,*1992; Lewin *et al.,* 1993). ALP appears in very high concentrations (>10,000 IU/L) in canine epididymal fluid whereas, the prostate and testis contain very small amounts of ALP. Because of its high concentration relative to that found in serum and prostatic fluid, seminal ALP functions as a useful marker of the sperm-rich fraction of the ejaculate (Kutzler *et al.,* 2005). Unilateral or bilateral occlusion of the ductal network or the efferent epididymal deferent could lead to azoospermia and oligospermia even in dogs with normal gonadal function thereby resulting in subfertility or infertility (Meyers-Wallen, 1995). Estimation of ALP in seminal plasma of azoospermic dogs has been suggested as a diagnostic aid to determine tubular patency (Olson *et al.,*1992; Meyers-Wallen, 1995). The present study was designed with an attempt to differentiate fertile and subfertile dogs on the basis of the values of seminal ALP in sperm-rich fraction of semen.

Materials and methods

Twenty dogs aged between three to six years with body weight ranging from 15 to 60 kg, presented for breeding soundness evaluation (BSE) at the University Veterinary Hospitals, Mannuthy and Kokkalai were chosen for the study. Complete breeding history of the stud dog was collected with special emphasis on the previous six months. Semen was collected by digital manipulation (Simon, 1997). Three collection were made two weeks apart. Immediately after collection of the sperm-rich fraction of the semen, initial sperm motility was recorded. The sperm concentration was analysed using the Neubauer hemocytometer. Percentage of live sperm and sperm abnormalities were recorded by Eosin and Nigrosine staining technique.

These animals were allotted into two groups *viz*., Group I (fertile group) and Group II (subfertile group) based on the previous six months breeding history and semen quality. Breed-wise distribution of the stud dogs under the present study were as following; German Shepherd (n=8, 40%), Pug (n=5, 25%), Labrador (LB) (n=2, 10 %), Beagle (n=1, 5 %), French bull dog (n=1, 5 %), Rajapalyam (n=1, 5 %), Golden Retriever (n=1, 5 %) and St. Bernard (n=1, 5 %).

Semen from these dogs was collected by digital manipulation and volume of various fractions were recorded. The second fraction was centrifuged at 1000 × g for 15 min at room temperature and the seminal plasma was removed from the sperm pellet. The separated seminal plasma was further clarified by centrifuging at 10,000 × g for 10 min at room temperature (Strzezek *et al.,* 2015). The seminal plasma separated thus was used for estimation of ALP. It was analysed using a semi-automatic analyser (MasterT biochemistry analyser, Hospitex diagnostics, Italy) and recorded in international unit per litre (IU/L).

Results and discussion

In the present study, the mean volume of the first, second and third fractions of semen ejaculate in Groups I and II was recorded as 1.17 ± 0.07, 1.55 ± 0.13, 11.3 ± 1.22 and 1.56 ± 0.37, 1.41 ± 0.35 and 8.46 ± 0.33 mL, respectively. No significant difference ($p>0.05$) was observed in the volume of the various semen fractions between the groups (Table 1). Our observation was in accordance with those of Kustritz (2007) who reported that volume of sperm-rich fraction ranged between 0.5 to 2.0 mL. The author had further opined that although total sperm output is considered as an indicator of semen quality, the volume may not directly represent the same. However, the volume is helpful in estimating total spermatozoa output per ejaculate which of course has a binding to semen quality.

The volume of prostatic fluid or third

fraction of semen in the present study was in agreement with the findings of Tesi *et al.* (2018) and Blendinger (2007) who had reported variations in the volume of prostatic fluid collected and that it depended on how much quantity was collected (how long the manual collection procedure lasted) and the size of the dog. These authors also observed that the volume of prostatic fluid ranged between 2 to 20 mL (average 5 mL).

The mean progressive motility of sperm in Groups I (84.17 ± 1.54 %) was found to be significantly higher ($p<0.01$) than those in Group II (45.71 ± 3.47 %, Table 2). The total motile sperm in normal canine ejaculates is between 70 and 90 per cent (Johnston *et al.,* 2001; Iguer-Ouada and Verstegen, 2001), although it may be lower after prolonged periods of sexual rest. It has been proposed that fertile dogs should have at least 70 per cent of total sperm motility (Larsen 1980).

The mean sperm concentration of second fraction of semen ejaculates in Groups I and II showed a highly significant difference (418.33 ± 36.64 and 112.07 ± 24.36 million/mL, $p<0.01$, Table 2). The mean total sperm output of second fraction of semen ejaculates, calculated on the basis of volume of the sperm-rich fraction, in Groups I and II was 645.11±98.43 and 190.24±32.45 million, respectively. On statistical analysis, a highly significant difference ($p<0.01$) was noticed between the groups (Table 2).

The total sperm count in the present study is in accordance with Johnston (1991) who reported that total sperm count in normal ejaculates may range from 300 to 2000 million. Freshman (2002) reported that average sized dogs should produce at least 250 to 300 million sperm/ ejaculate. Although 12 among 14 dogs in Group II were medium to large sized dogs, the total sperm output was significantly lower than these proposed average values. Martinez (2004) opined that such lower sperm concentration requires further investigation to differentiate incomplete ejaculation or azoospermia due to testicle related issues by estimating ALP.

Table 1. Comparison of volume of various fractions of semen in fertile and subfertile groups (in mL)

Parameter	Fertile group (n=6)	Subfertile group (n=14)	t-value	p-value
F1	1.17 ± 0.07^{b}	1.56 ± 0.37^{b}	0.236^{ns}	0.816
F2	1.55 ± 0.13^{b}	1.41 ± 0.35^{b}	0.572^{ns}	0.574
F3	11.3 ± 1.22^{a}	8.46 ± 0.33^{a}	1.410^{ns}	0.175
F-value	75.363**	215.275**		
P-value	<0.001	<0.001		

*** Significant at 0.01 level (p<0.01)*

Table 2. Comparison of semen parameters between fertile and subfertile groups

Parameter	Fertile group (n=6)	Subfertile group (n=14)	t-value	p-value
Progressive motility (%)	84.17 ± 1.54	45.71 ± 3.47	10.13**	<0.001
Concentration (million/mL)	418.33 ± 36.64	112.07 ± 24.36	6.915**	< 0.001
Total Sperm Output (million)	645.11±98.43	190.24±32.45	7.133**	< 0.001
Live (%)	84.92 ± 1.56	49.54 ± 2.35	12.562**	< 0.001
Abnormality (%)	6.50 ± 0.99	11.50 ± 1.56	2.003	0.060

*** Significant at 0.01 level (P<0.01); ns non-significant (P>0.05)*

Table 3. Comparison of ALP of sperm-rich fraction between fertile and subfertile group

Parameter	Fertile group (n=6)	Subfertile group (n=14)	t-value	P-value
ALP (IU/L)	168121.67 ± 22443.25	49605.86 ± 12669.68	4.895**	< 0.001

*** Significant at 0.01 level (P<0.01); ns- non-significant (P>0.05)*

The mean total viable sperm in semen ejaculates in Groups I and II was recorded to be 84.92 ± 1.56 and 49.54 ± 2.35 per cent, respectively, which differed significantly between groups ($p<0.01$, Table 2). The mean percentage of total sperm abnormality of semen ejaculates was 6.50 ± 0.99 and 11.50 ± 1.56 per cent, respectively in in Groups I and II with no significance of difference between groups. Normal canine ejaculates should have at least 80 per cent morphologically normal and viable spermatozoa (Johnston *et al.,* 2001). When the proportion of morphologically normal spermatozoa was below 60 per cent, fertility was found to be adversely affected (Oettlé, 1993). Thus, it is evident that dogs in Group I were fertile, while those in Group II were subfertile.

Tesi *et al.* (2018) reported that progressive motility, total spermatozoa output and the percentage of morphologically normal viable spermatozoa in the semen used for artificial inseminations (AI) resulting in a pregnancy were 83.9 per cent, 627.6 $\times 10^6$ and 64.9 per cent, respectively. In case of unsuccessful AIs, these values were significantly lower and were respectively, 66.5 per cent, 389.4 $\times 10^6$ and 42 per cent, respectively ($p<0.05$). In the present study, semen parameters like sperm concentration, total sperm output, progressive motility and live sperm percentage were significantly higher in Group I than Group II.

The mean ALP (IU/L) in Groups I and II was recorded as 168121.67 ± 22443.25 and 49605.86 ± 12669.68, respectively. On statistical analysis, a highly significant difference ($p<0.01$) was noticed between the groups (Table 3).

ALP is an enzyme with dephosphorylation function present in various organs like bone, liver and intestinal tissue. Mann (1964) opined that ALP of semen had specific role in sperm glycolytic pathway and fructose formation which facilitated in providing a source of energy to spermatozoa. Kutzler (2005) reported that although the biochemical role of ALP in the seminal plasma is not fully clear, in alkaline pH it could have a role in the glycolytic pathway by hydrolysis of monophosphate esters such as fructose 1-phosphate, fructose 6-phosphate and fructose 1,6-diphosphate.

Frenette *et al.* (1986) observed that in seminal plasma of canine ALP was mainly produced by epididymis and not in the prostate. A differential diagnosis of incomplete ejaculation and azoospermia in dogs is thus possible by estimating the ALP in the canine seminal plasma wherein a reduced concentration (< 5000 IU/L) of ALP in the seminal plasma suggested bilateral occlusion of ductal network (epididymis or vas deferens) (Gobello *et al.,* 2002). Kutzler (2005) performed immuno-histochemistry in canine testis and epididymis and identified the activity of seminal ALP in the epithelial cells of the head, body and tail of the epididymis as well as in the seminiferous tubules of the testis.

In the present study, semen parameters like progressive motility, sperm concentration, total sperm output and live sperm percentage were significantly more in the Group I than Group II. Sperm rich fraction was used exclusively for estimation of the ALP. Though the concentration of ALP was more than 40,000 IU/L in all the dogs under the present study (indicating a complete ejaculation in both the groups), it was significantly higher in fertile dogs, suggesting a possible relation to fertility. Johnston *et al.* (2001) reported that ALP should be greater than 5,000 IU/L in seminal plasma and further observed that ALP in dog with gonadal causes like azoospermia ranged from 5,500 to 81,600 U/L. Bucci *et al.* (2014) opined that the ALP function is still not clear, though it may serve as a decapacitating factor. No studies could be traced out, which had compared ALP between fertile and subfertile dogs.

Conclusion

Significantly higher ALP values in sperm-rich fraction in fertile dogs when compared to subfertile dogs could potentially be of use to serve as a marker of fertility in stud dogs. Although the concentration of ALP was more than the proposed cut off value for incomplete ejaculation in all the dogs in the present study, it however suggested a possible relation to fertility. Further studies involving greater number of animals and specific and

critical analysis regarding the role of ALP on fertility in stud dogs would be required before confirming its practical utility.

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The authors are thankful to the Dean of College of Veterinary and Animal Sciences, Mannuthy for the facilities provided for the conduct of the research. The first author acknowledges the fellowship provided by the Kerala Veterinary and Animal Sciences University for the academic and research programme.

Conflict of interest

The authors declare that they have no conflict of interest.

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Histomorphological studies on the testicular capsule of crossbred Large White Yorkshire boars (*Sus scrofa domesticus*)

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Abstract

The present study was conducted on the testes of six healthy adult Large White Yorkshire pigs. Parenchyma of testes of adult boars was surrounded by a thick fibrous testicular capsule composed of inner tunica vasculosa, middle tunica albuginea, and outer visceral layer of tunica vaginalis. Tunica vasculosa was prominent and characterised by loose connective tissue contained numerous blood vessels. Tunica albuginea was composed of irregular connective tissues made up of collagen, elastin and reticular fibres. Visceral layer of tunica vaginalis was found as outer thin layer of testicular capsule comprised of mesothelium and connective tissue layer. Connective tissue layer was blended with underlying connective tissues of tunica albuginea.

Keywords: *Histomorphology, pig, testicular capsule*

According to Leena *et al.* (2004), only a few states in India are successful in modern pig farming, with Kerala being the most notable. Availability of plenty of swill and food habits, makes pig farming a beneficial livestock sector in Kerala (Kannan *et al.*, 2008). Testes are the primary reproductive and endocrine organs or gonads in the male, responsible for the production of functional spermatozoa, secretion of hormones and factors that involve all aspects of sexual development and reproductive functions. Studies and literature pertaining to histomorphology of testes of pigs are scanty, hence the study was undertaken.

#Part of the MVSc thesis of the first author submitted to the Kerala Veterinary and Animal Sciences University, Pookode, Wayanad, Kerala

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Materials and methods

The present study was conducted on the testes of six healthy adult Large White Yorkshire (LWY) pigs slaughtered at the Meat Technology Unit, Kerala Veterinary and Animal Sciences University. Tissue samples of testes were collected from cranial, middle and caudal parts of the testes and fixed in 10% neutral buffered formalin for 48 hours. Paraffin blocks were made by alcohol-xylene sequence (Luna, 1968). The sections were cut at 5µ thickness and stained by Haematoxylin and eosin (H&E) staining for general cytoarchitectural studies (Luna, 1968). Special staining methods like Van Gieson's staining for collagen fibres (Luna, 1968), Masson's trichrome staining for muscles and collagen fibres (Luna, 1968), Verhoeff's staining (Singh and Sulochana, 1996) for elastic fibres, Gomori's method for reticular fibres (Luna, 1968) and Phosphotungstic acid haematoxylin (PTAH) staining for muscles (Luna, 1968) were also employed.

Results and discussion

Testicles of crossbred Large White Yorkshire boars were found white in colour, large, regularly elliptical shaped, placed at the perineal region and directed backwards and upwards forming a prominent landmark about 6-8 cm below the anus. Parenchyma of the testes of adult boars was surrounded by a thick fibrous testicular capsule and was characterised by regularly arranged dense connective tissue, made up of three layers *viz.,* inner tunica vasculosa, middle tunica albuginea and outer visceral layer of tunica vaginalis (Fig. 1). These findings were similar to the reports of Ahmed and Sinowatz (2005) in bull, Dhyana *et al.* (2016), Singh *et al*. (2019) and Singh *et al*. (2020) in adult boar and Khan *et al.* (2019) in buck.

The innermost, highly vascularised and prominent tunica vasculosa, which was contained in the deeper portion of the capsule was distinguished by dense regular connective tissue (Fig. 1). These observations are in accordance with the reports of Shukla *et al*. (2013) in stallion and Dhyana *et al.* (2016), Singh *et al*. (2019), Singh *et al*. (2020) in adult boar. Tunica albuginea, the middle layer of testicular capsule had dense regular connective tissue which was composed of collagen (Fig. 2), elastic (Fig. 2) and reticular fibres (Fig. 3) along with fibroblasts, smooth muscle fibres (Fig. 4) and a few blood vessels. These observations are similar to the reports given by Shukla *et al*. (2013) in stallion, Dhyana *et al.* (2016) in domestic pig of Hyderabad, Singh *et al*. (2019) in local pig (Zovawk) of Mizoram and Singh *et al*. (2020) in LWY boar and contrary to the findings of Trautmann and Fiebiger (1952), who discovered tunica vasculosa in the centre of the testicular capsule in both stallion and boar.

The inner layer of capsule continued as inconspicuous connective tissue trabeculae and divided the parenchyma of the testis into several inconspicuous lobules (Fig. 5). Trabeculae were observed as thick strands of collagen and elastic fibres, supplied with blood and lymph vessels. These observations are in accordance with the previous reports of Al-Kelaby *et al*. (2017) in ram and buck and Dhyana *et al.* (2016) and Singh *et al*. (2020) in boar but are not in agreement to the observations of Ahmed and Sinowatz (2005) in bull and Shukla *et al*. (2013) in stallion, who found that the connective tissue structure was not as distinct and thus lobulation was insignificant.

Visceral layer of tunica vaginalis was found as a thin outer layer of testicular capsule comprised of outermost mesothelium and underlying connective tissue layer (Fig. 1). Connective tissue layer was formed of collagen fibres and a few elastic and reticular fibres that blended with underlying connective tissues of tunica albuginea as observed as Dhyana *et al.* (2016) in pigs. Due to this blending, visceral layer of tunica vaginalis could not be clearly delineated from the outer fibrous layer of testicular capsule

Conclusion

The results presented on the histology of testicular capsule of boars are those of normal, adult, sexually active ones. Parenchyma of the testes of adult boars was surrounded by a thick fibrous testicular capsule and was characterised by regularly arranged dense connective made up of three layers *viz.,* inner tunica vasculosa, middle tunica albuginea and outer visceral

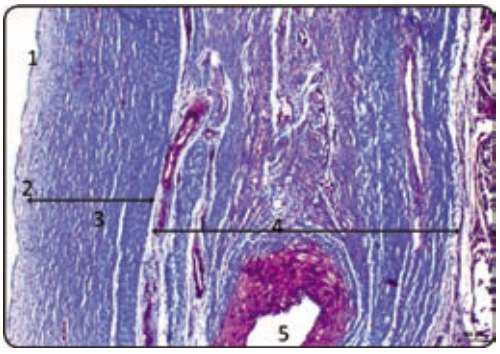

Fig. 1. Cross section of testicular capsule with visceral layer of tunica vaginalis. Masson's trichrome method x 100

1. Mesothelium,
2. Visceral layer of tunica vaginalis,
3. Tunica albuginea,
4. Tunica vasculosa, 5. Artery

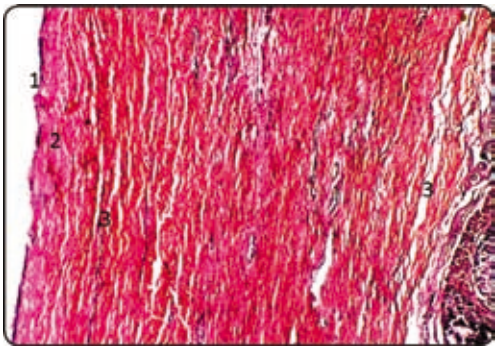

Fig. 2. Cross section of testicular capsule with visceral layer of tunica vaginalis.

Verhoeff's elastic stain x 100

1. Mesothelium, 2. Collagen fibres,

3. Elastic fibres

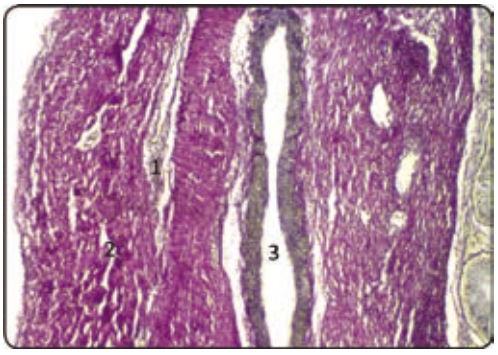

Fig. 3. Cross section of testicular capsule with visceral layer of tunica vaginalis. Gomori's method for reticular fibres x 100

1. Reticular fibres, 2. Collagen fibres,

3. Artery

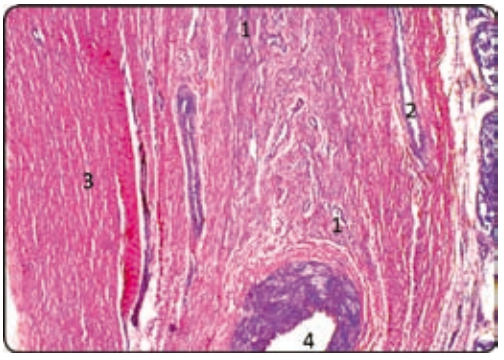

Fig. 4. Cross section of testicular capsule with visceral layer of tunica vaginalis. PTAH method for muscles x 100

1. Smooth muscle, 2. Vein, 3. Collagen fibres, 4. Artery

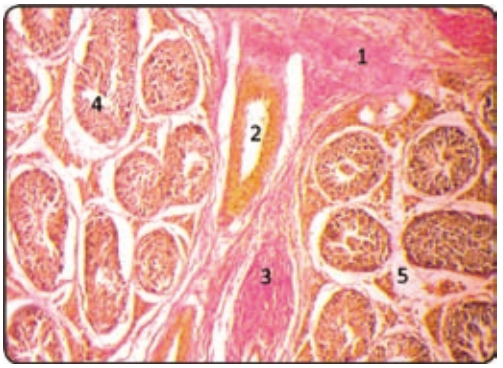

Fig. 5. Cross section of testis showing origin of trabecula from testicular capsule.

van Gieson's method x 200

1. Tunica vasculosa, 2. Artery, 3. Trabecula,

4. Seminiferous tubule, 5. Testicular lobule

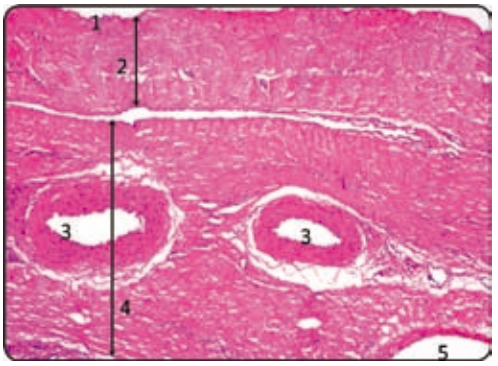

Fig. 6. Cross section of testicular capsule with visceral layer of tunica vaginalis. H&E x 100.

1. Mesothelium, 2. Fibrous layer, 3. Artery,

4. Vascular layer, 5. Vein

layer of tunica vaginalis. Tunica albuginea was the middle layer of testicular capsule which was composed of collagen, elastic and reticular fibres along with fibroblasts, smooth muscle fibres and a few blood vessels. The presence of smooth muscle cells in the capsule could be attributed to the transport of spermatozoa whereas the collagen fibres and fibroblasts for the mechanical support.

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Conflict of interest

The authors declare that they have no conflict of interest.

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RESEARCH ARTICLE
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Radiographic evaluation of hoof affections in dairy cattle

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Abstract

The study was undertaken to identify radiographic changes of hoof affections in dairy cattle. Out of the 78 cases radiographed, 67 cases were found to have lesions. Lateral and dorso-palmar/ plantar views of the affected foot of selected animals under study was evaluated. Soft tissue mass at inter-digital space, alterations in bone density of third phalanx, deviation of third phalanx were the major radiographic lesions diagnosed followed by fracture of third phalanx, osteomyelitis, deviation of second phalanx, periosteal reactions on phalanges and soft tissue swelling at coronary region. No radiographic changes were present in 14.10 per cent of the cases.

Keywords*: Radiography, hoof, dairy cattle*

Lameness is a disease of high producers which can cause a significant loss of milk yield (Mohamdnia and Khaghani, 2013). It is an ever-persistent issue and risk to the productivity and indirectly affect the profit of dairy farmers. Not only it shortens the productive life of cows, but also it adds many hours of labour in terms of its treatment (Mahendran and Bell, 2015). Since lameness is painful condition and causes financial loss to the farmers, it is considered as a serious welfare issue (Kossaibati and Esslemont, 1997).

Lameness is a multifactorial disease, a combination of several disorders in limbs and affections of hoof. Majority of the lameness cases reported are due to lesions in the feet; hind foot had more lesions compared to front foot (Shearer, 1997). Anju *et al.* (2019) atudied about the morphology and morphometry of buffalo hooves and reported about the normal morphometry,

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Early diagnosis and implementing appropriate treatment based on type of hoof affections are essential in sustainable reduction of lameness cases in cattle.

Radiographic diagnosis can be an effective tool for the differential diagnosis of hoof disorders. Most of the hoof disorders are associated with marked rotation or sinking of pedal bone and it cause significant changes in hoof dimensions and radiographic analysis aids in decision making on treatment plans and to predict the prognosis of lameness cases.

Materials and methods

The present study was conducted in selected organised dairy farms and small holder livestock units. Animals were observed at rest and during progression at different paces for evaluation of lameness. Those animals which showed moderate to severe lameness were suspected for osseous lesions and radiographically evaluated. The hooves were radiographed in dorso-palmar (forelimbs) or dorso-plantar (hind limbs), oblique and lateral views. Radiographs were examined and radiographic changes were recorded. Radiographs of hooves that did not manifest alterations were used as controls for comparison with affected radiographs. Radiographs were taken using a portable X-ray machine EP CORSA 2.4 with 8 MAS and 50 KVP settings.

Results and discussion

Lateral and dorso-palmar or dorso-plantar radiographs of the affected foot of animals under study was taken. A total of 78 animals were radiographed of which 67 animals showed radiographic lesions. Lesions that could be detected were periosteal reactions on phalanges (2.56 per cent) (Fig.1), osteomyelitis (2.56 per cent) (Fig. 2), alterations in bone density of third phalanx (21.79 per cent) (Fig. 3), fracture of third phalanx (7.69 per cent) (Fig.5), deviation of third phalanx (21.79 per cent) (Fig. 6), deviation of second phalanx (2.56 per cent) (Fig. 4), soft tissue mass at interdigital space (25.64 per cent) and soft tissue swelling at coronary region (1.28 per cent). Details of radiographic lesions observed during this study is tabulated (Table 1).

Lateral and dorso-palmar or dorso-plantar radiographic views were taken in the present study similar to that used by Farrow (1999). Radiography is the best way to differentially diagnose the foot affections (Nouri *et al.*, 2011). In a radiographic study of hoof affections Parizi and Shakeri (2007), identified osteomyelitis, exostosis, deformity of bones, change in bone density, soft tissue calcification, arthritis, degenerative joint diseases, ankylosis of joint, rotation of phalanges, displacement of navicular bone and fracture were obtained. One case of abscess at coronary region was radiographed and extend of infection into the distal interphalangeal joint was identified. Radiographic study of distal interphalangeal joint is useful in identifying the extent and duration of sepsis of joint and bones (Anderson *et al.,* 2017).

Out of the ten corkscrew claws radiographed, eight claws were found to have deviation of third phalanx. In one case lateral deviation of second phalanx could be noticed. In corkscrew claws, upward and inward rotation of the toe region led to weight bearing on abaxial wall of hoof and sole become completely non-weight bearing (Amstel, 2017). This rotation and imbalance from the normal biomechanics of weight bearing may be the reason for higher incidence of deviation in third phalanx in cork screw claws. Nouri *et al.* (2011) observed structural alterations in phalanx in animals with chronic hoof affections when they were evaluated radiographically. Radiography of nine cases with sole ulcers found to have deviation of third phalanx. Excessive overgrowth of hooves at the toe lead to deviation of pedal bone and pinching of the deviated pedal bone in hoof corium led to incidence of sole ulcer (Blowey, 1993). Radiography helped to assess the position of pedal bone and supporting structure of the hoof. Most of the hoof disorders are associated with marked rotation or sinking of pedal bone (Philip, 2018). Shearer and Amstel (2017) opined radiographs are indicated in deep seated penetration of foreign body into the sole to rule out a fracture. Six cases of pedal bone fracture were diagnosed by radiography. Early radiographic diagnosis of hoof disorders helps to make decisions on treatment plans and to predict the prognosis (Philip, 2018).

Table 1. Radiographic findings of different hoof affections

Type of lesions	Forelimb	Hindlimb	Total	
	No. of cases	No. of cases	No. of cases	%
Periosteal reaction on phalanges	-	2	2	2.56
Osteomyelitis	2	-	2	2.56
Alteration in bone density of third phalanx	3	14	17	21.79
Fracture of third phalanx	2	4	6	7.69
Deviation of third phalanx	2	15	17	21.79
Deviation of second phalanx	-	2	2	2.56
Soft tissue mass at interdigital space	2	18	20	25.64
Soft tissue swelling at coronary region	-	1	1	1.28
No abnormalities detected	3	8	11	14.10
Total	14	64	78	100

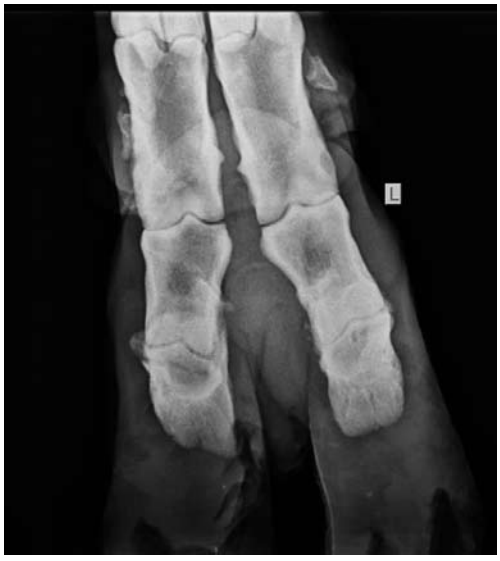

Fig. 1. Periosteal reactions

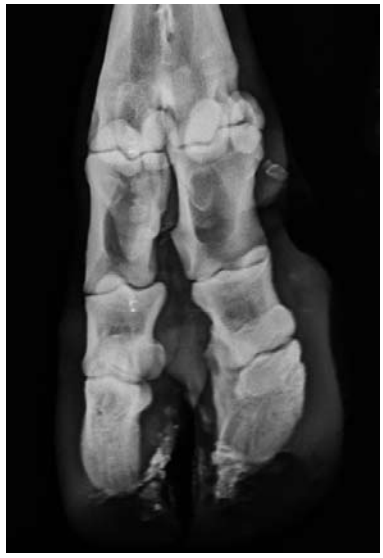

Fig.2. Osteomyelitis

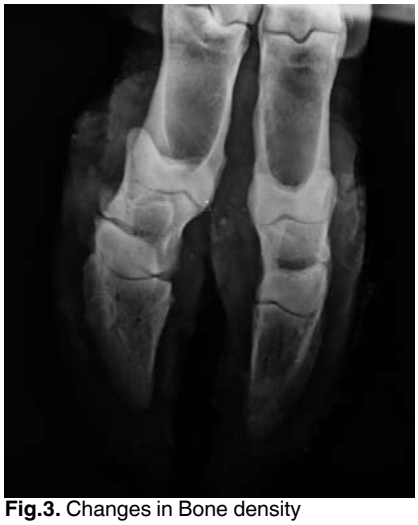

Fig.3. Changes in Bone density

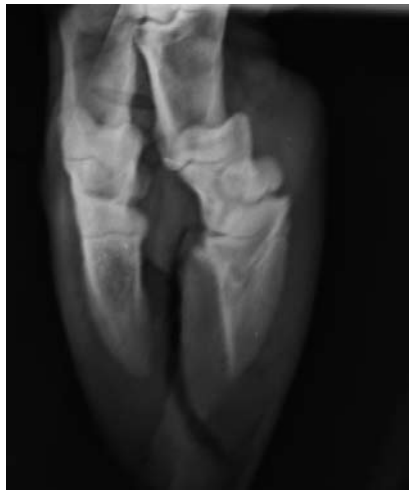

Fig.4. P2 deviation

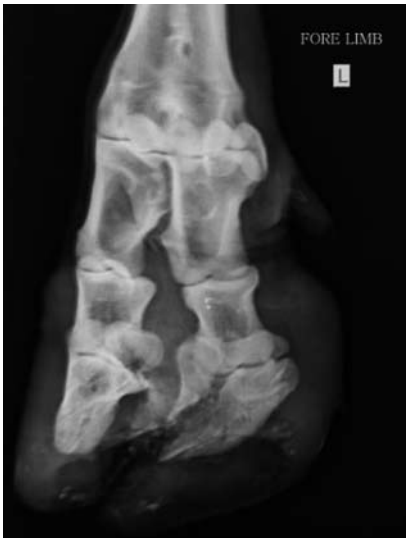

Fig. 5. Fracture of 3rd Phalanx

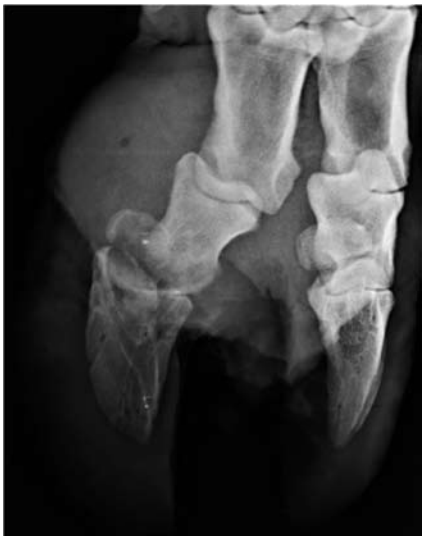

Fig.6. P3 deviation

Conclusion

From the present study it could be understood that lameness originating from the hoof should not be ignored and affected animals should undergo detailed investigations to diagnose the actual cause of lameness. Radiography ccould be an effective tool for the differential diagnosis of hoof disorders. Most of the hoof disorders are associated with marked rotation or sinking of pedal bone and it cause significant changes in hoof dimensions and radiographic analysis aids in decision making on treatment plans and to predict the prognosis of lameness cases.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Tetracycline efflux pump genes in *Escherichia coli* from retail chicken in central Kerala

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Abstract

The rearing of chicken in India has undergone a drastic change from backyard production to commercial intensive farming. This has led to the use of antibiotics in therapy, metaphylaxis and as growth promoters. Escherichia coli are commensals that inhabit the gut of man and animals. The detection of virulent pathotypes of E. coli in chicken is a huge threat to human health. The present study assessed 200 retail chicken sold in central Kerala for the presence of virulent E. coli and studied the tetracycline susceptibility followed by detection of tetA and tetB gene. E. coli was detected in 64 per cent of the samples. The virulence genes, eaeA and aggR were detected in 52.2 and 3.9 per cent of the E. coli isolates, respectively. Tetracycline resistance by antibiotic susceptibility testing (ABST) was found in 30.84 per cent of the virulent isolates. The tetracycline efflux pump protein coding genes, tetA and tetB were detected in 56.67 and 25 per cent, respectively. The detection of drug resistant bacteria is a threat to public health as tetracycline is classified as a highly important antibiotic in human medicine.

Keywords: *E. coli, tetracycline resistance, tetA, tetB*

Chicken is a cheap source of animal protein and it is relished because of its versatility in cuisine and because no religious or cultural taboos are associated with its consumption (Khara *et al.,* 2020). In order to meet the growing demand, chicken rearing in India has undergone a paradigm shift from mere backyard rearing to a commercial enterprise. In order to cater to the boost in demand, antibiotics are being rampantly used in metaphylaxis, therapy and also in feed as growth promoters. Promiscuous use of antibiotics leads to increase in the incidence of drug resistant bacteria.

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Escherichia coli are commensals which reside in the gut of poultry. Improper dressing practices leads to the contamination of chicken with faecal matter thereby contributing to the presence of *E. coli* in the meat. Most of the strains of *E. coli* are usually harmless but a few strains are highly pathogenic. *Escherichia coli* strains that cause enteric disease in their hosts are called diarrhoeagenic *E. coli* (DEC) and their pathogenesis is associated with a number of virulence attributes which varies with the pathotypes (Xia *et al.,* 2010). Intimin is a 94-kDa outer membrane protein encoded by the *eae*A gene which is required for the intimate attachment of bacteria with the enterocyte membranes (Trabulsi *et al*., 2002). The *agg*R gene is a transcriptional regulator which regulates the expression of aggregative adherence fimbriae in enteroaggregative *E. coli*. Penetration into the epithelial cells and dissemination from cell to cell in enteroinvasive *E. coli* are mediated by an invasion-associated locus (*ial*), located on a plasmid and the invasion plasmid antigen H (*ipaH*) genes present in both chromosome and plasmid (Farajzadeh-Sheikh *et al*., 2020).These organisms are notorious for their role in the transmission of antimicrobial resistance (AMR) as faecal microbes acts as reservoirs of AMR (Purohit *et al.,* 2019).These organisms are notorious for their role in the transmission of antimicrobial resistance (AMR) as faecal microbes acts as reservoirs of AMR (Purohit *et al.,* 2019).

Tetracyclines are broad spectrum antibiotic and are popular because of their efficacy and low cost (Zibandeh *et al*., 2016). They are used in therapy and also as growth promoters as they have been proven to increase the weight of the birds. The first generation tetracyclines such as oxytetracycline and chlortetracycline have been used as growth promoters for decades. Second generation tetracyclines such as minocycline and doxycyline have been used in metaphylaxis and therapy. The indiscriminate use of tetracycline has led to the selection of tetracycline resistant bacteria (Koo and Woo, 2011). The present study was envisaged with the objective of studying the prevalence of *E. coli* in retail chicken sold in central Kerala, occurrence of virulence genes, assess the susceptibility of the isolates to tetracycline and finally to check if the isolates harbour the efflux pump encoding genes, *tet*A and *tet*B.

Materials and methods

Hundred retail chicken samples each were collected from two central districts of Kerala *viz.*, Thrissur and Ernakulam. Each sample of chicken consisted of 250g and included portions of neck, breast and thigh (Bhandari *et al*., 2013). The chicken samples were brought to the laboratory immediately under refrigeration conditions. The isolation of *E. coli* was done according to the procedure of Meng *et al.* (2001). Briefly, from each pooled composite chicken meat sample, a 25 g portion was aseptically removed using sterile scissors and forceps. The sample was transferred to 225 mL volumes of buffered peptone water (Difco) in sterile stomacher bags, processed for 120 s. in a stomacher (Smasher, AES, France) and incubated at 37°C for 18 h. Following incubation, a loopful was transferred to Mac Conkey agar and incubated at 37°C for 24 h. At the end of incubation, lactose fermenting bright pink coloured colonies surrounded by bile precipitate was selected based on morphology and subjected to biochemical tests as described by Barrow and Feltham (1993). The isolates confirmed as *E. coli* by culture were subjected to snap chill method for the extraction of DNA (Swetha *et al*., 2015).

The DNA was subjected to PCR for the detection of virulence genes, *eae*A, *agg*R and *ipa*H using the primers as shown in Table 1. Multiplex PCR was performed in a final volume of 25 µL reaction mixture using 3 µL of extracted DNA as template. The cyclic conditions were standardised in the study: initial denaturation at 95°C for 5 min. followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min, extension at 72°C at 40 s followed by final extension at 72°C for 10 min.

All the isolates that were positive for virulence genes were subjected to antibiotic susceptibility test against tetracycline using standard disc diffusion method (Skočková *et al*., 2015) using tetracycline disc (HiMedia)

with a concentration of 30µg. The isolates that showed resistance to tetracycline by phenotypic method were subjected to two separate PCR for the detection of *tet*A and *tet*B genes. The reaction mixture included, 2.5 µL of 10X PCR buffer, 2 µL of 25m*M* $MgCl_2$, 0.50 µL of *Taq* DNA polymerase (5Units/µL), 0.50 µL of dNTP Mix (10m*M*), 10 pmoles/µL each of forward and reverse primers of *tet*A and *tet*B and nuclease free water made upto 25 µL total volume. The primers used for the detection of *tet*A and *tet*B are shown in Table 2.

The cyclic condition of PCR for *tet*A gene included an initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 59.5°C for 1 min, extension at 72°C for 1 min followed by final extension at 72°C at 5 min. The cyclic conditions followed for *tet*B gene was as follows; initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 66°C for 30 s, extension at 72°C for 45 s which was followed by final extension at 72°C for 5 min.

Results and discussion

The prevalence of *E. coli* by culture was found to be 67 and 61 per cent from Thrissur and Ernakulam, respectively, with an overall prevalence of the organism from central Kerala being 64 per cent. The results are similar to studies in Bangladesh, where a prevalence of 63.5 per cent was reported (Rahman *et al.*, 2020). However, a higher prevalence of 87.5 per cent was reported by Eyi and Arslan (2012) from Turkey and an occurrence of 76.5 per cent was reported from coacal swabs of chicken from Kollam district of Kerala (Afsal *et al*., 2021). A study from Wayanad in Kerala reported the occurrence of *E. coli* in 36.11 per cent of cloacal swabs from broiler chicken which is much lower than the present study (Sathya *et al*., 2019). There was no significant statistical difference ($p \geq 0.01$) in the prevalence of the organism between the two districts.

Of the three virulence genes, only *eae*A and *agg*R were detected. None of the isolates harboured *ipa*H gene. The amplicons were obtained at 209 and 254bp for *eae*A and *agg*R respectively (Fig. 1). The *eae*A gene was detected in 86.57 and 77.05 from Thrissur and Ernakulam, respectively. Totally, the gene was detected in 81.81 per cent of the isolates. This accounts for the presence of *eae*A in 52.2 per cent of the retail chicken tested. This is in accordance with the study by Wang *et al.* (2017), where *eae*A was detected in 50 per cent of the chicken tested in Japan. The *agg*R gene was detected from 2.99 and 4.92 per cent of the *E. coli* isolates from Thrissur and Ernakulam. Overall, *agg*R was detected in 3.9 per cent of the isolates. However, a higher per cent of occurrence of *agg*R (24.44) was detected by Kagambega *et al*. (2012) from West Africa. A study from Mumbai in India by Godambe *et al*. (2017) could not detect either *eae*A or *agg*R from any of the *E. coli* isolates from chicken.

Of the 107 isolates of virulent *E. coli* obtained from central Kerala, resistance to tetracycline was detected in 33 isolates (30.84 per cent), whereas intermediate susceptibility was noted in 27 isolates (25.23 per cent). The results are in accordance with that of Skočková *et al*. (2015), from Czech Republic, where the level of resistance to tetracycline was reported to be 34.5 per cent. However, studies by Ingram *et al*. (2013), Chakravarty *et al*. (2015) and Bhardwaj *et al*. (2021) from Western Australia and different parts of India, respectively reported cent per cent resistance to tetracycline. Both the tetracycline resistant and intermediate susceptible isolates were subjected to PCR for the detection of *tet*A and *tet*B genes. The amplicons for *tet*A and *tet*B were obtained at 209 and 169 bp, respectively (Fig. 2). The district-wise distribution of the tetracycline resistance genes *tet*A and *tet*B is shown in Table 3. The *tet*A gene was detected in 75.76 per cent and 33.33 per cent of the resistant and intermediate susceptible isolates, respectively. Whereas, *tet*B was detected in 39.39 and 7.40 per cent of resistant and intermediate isolates, respectively. Both *tet*A and *tet*B together were detected in 30.30 per cent of resistant and 3.70 per cent of intermediate susceptible isolates. Overall *tet*A was detected in 56.67 per cent which is in accordance with a study in Norway where *tet*A was detected in 55 per cent of resistant isolates (Sunde and Norström, 2006). Nevertheless, a higher level of detection of 89.5 and 74 per cent was reported by Van *et al*. (2008) and Bhardwaj

Table 1. Primers used for the virulence genes of *E. coli*

Primer	Primer sequence	Size (bp)	Reference
*eae*A F	5'- TCCTGGTTCCCTTATCAACG-3'	209	In-house designed
*eae*A R	5'- GCGACCGCTACCAACATAG-3'		
*agg*R F	5'-GTATACACAAAAGAAGGAAGC-3'	254	Ratchtrachenchai *et al.* (1997)
*agg*R R	5'-ACAGAATCGTCAGCATCAGC-3'		
*ipa*H F	5' -TCACATTGCCCATTTGTACG- 3'	295	In-house designed
*ipa*H R	5' -GCAGAGACGGTATCGGAAAG-3'		

Table 2. Primers used for the identification of *tet*A and *tet*B genes

Primer	Primer sequence	Size (bp)	Reference
*tet*A F	5'- F GCTACATCCTGCTTGCCTTC-3'	209	In-house designed
*tet*A *R*	5'- ATAGATCGCCGTGAAGAGGA -3'		
*tet*B F	5'- TCAGCGCAATTGATAGGCCA -3'	169	In-house designed
*tet*B R	5'- TTTCGCCCCATTTAGTGGCT -3'		

Table 3. District – wise distribution of *tet* genes

Sl. No.	District	Resistant isolates							Intermediate susceptibility							
		No. of isolates	*tet*A		*tet*B		*tet*A and *tet*B		No. of isolates	*tet*A		*tet*B		*tet*A and *tet*B		
			No.	%	No.	%	No.	%		No.	%	No.	%	No.	%	
1	Thrissur	15	11	73.33	1	6.67	1	6.67	20	7	35.00	2	10.00	1	5.00	
2	Ernakulam	18	14	77.78	12	66.67	9	50.00	7	2	28.57	0	0.00	0	0.00	
3	Total	33	25	75.76	13	39.39	10	30.30	27	9	33.33	2	7.40	1	3.70	

et al. (2021), respectively. The *tet*B gene was detected in 25 per cent of the tetracycline resistant and intermediary isolates. However a study in Korea (Koo and Woo, 2011) reported a higher occurrence of *tet*B gene (41.3 per cent). Only 13 per cent of the *E. coli* isolates obtained from chicken in Karnataka, India harboured *tet*B gene (Bhardwaj *et al*., 2021). The combined presence of both *tet*A and *tet*B was detected in 18.33 per cent of the isolates. This is contrary to a study in Vietnam, where both the genes were present at the same level of 89.5 per cent (Van *et al*., 2008).

Till date three resistance mechanisms to tetracycline have been recorded. The efflux pump proteins are one of the best studied mechanisms. These efflux pumps push the drug out of the bacterial cell using an energy dependent process. Twenty three different efflux pump genes are identified so far of which the most common ones being *tet*A and *tet*B. The *tet*A codes for resistance to tetracycline but not minocycline. On the other hand, *tet*B codes for resistance to both tetracycline and minocycline. Another mechanism of tetracycline resistance is by ribosomal protection coded by 11 genes. Yet another mechanism of resistance is by enzymatic inactivation of tetracycline by three genes (Roberts and Schwarz, 2017). The present study targeted the two most common genes coding for the efflux pump. Thus, not all the isolates that showed resistance to tetracycline by phenotypic methods were confirmed with the molecular tools.

The study highlights the presence of tetracycline resistant *E. coli* among healthy broiler chicken sold in central Kerala. The detection of drug resistant bacteria is a threat to public health as tetracycline especially chlortetracycline is classified as a highly important antibiotic to be reserved for use in humans and is listed as one of the critically important antibiotic published by the World Health Organisation (WHO, 2019). In order to

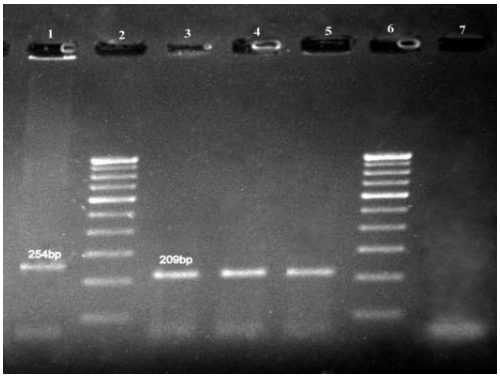

Fig. 1. Detection of virulence genes of *E. coli* by PCR.
Lane 1- *agg*R Lane 2 - 100 bp ladder Lane 3-5 – *eae*A Lane 6 - 100 bp ladder Lane 7 - Negative control

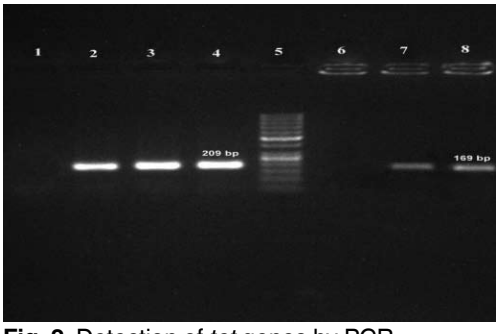

Fig. 2. Detection of *tet* genes by PCR

Lane 1 - Negative control *tet*A Lane 2 - 4 *tet*A Lane 5 - 50 bp ladder Lane 6 - negative control *tet*B Lane 7-8 – *tet*B

curtail the spread of antimicrobial resistance, a multisectoral and multifaceted one health approach must be adopted in addition to antibiotic stewardship, infection control and strict regulations on the use of antimicrobials.

Conclusion

Tetracycline is listed as an important antibiotic, because of its significance in human medicine. Confirmation of resistance against tetracycline in *E. coli* isolated from chicken is a great threat to public health. Furthermore, the organism is also known for the transfer of drug resistance genes not only within the same genera but also among other bacterial genera which again complicates the issue. Hence the results of the present study emphasises the need to use tetracycline with caution in poultry production so as to curb the spread of AMR through the food chain.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Study on personal profile of members in biodiversity management committees of Kerala

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Abstract

This is a study of the personal profile of members in Biodiversity Management Committees (BMCs), functioning under selected local self-governments of Kerala. BMCs constitute the grass root level institutional machinery for decentralized biodiversity governance. Five BMCs each from six districts, two each from the northern, central, and southern zones were selected purposively based on key informant technique. The selection of districts was based on criteria viz. indigenous cattle population, documentary evidence on native livestock of conservation value and expert guidance by subject matter specialists. Data regarding personal attributes of two hundred and forty members, eight each from thirty selected BMCs, procured through e-surveys and telephonic interviews were analyzed statistically. Results indicated dismal representation of women (37.08 per cent) as well as youth (21.25 per cent) in the committees. Another significant finding was the prevalence of political/ social workers among BMC members (61.66 per cent). The representation of stakeholder communities including those of farming, livestock and fisheries was minimal (17.08 per cent), whereas there was absolute absence of traditional knowledge holders and practitioners of indigenous medicine. Graduates and postgraduates together constituted nearly half of the respondents. All the members reported a working experience of five years in BMCs. The findings regarding training exposure in biodiversity management among BMC members revealed that a vast majority received not more than one training (79.58 per cent). Only a few had attended two or three training programmes (16.25 per cent). The Awards/recognitions received by individual members in biodiversity management was minimal (0.42 per cent), whereas at organizational level, 43.33 per cent of BMCs received only one and 3.33 per cent received more than one awards.

#Forms part of the MVSc thesis of the first author submitted to Kerala Veterinary and Animal Sciences University, Pookode, Wayanad, Kerala

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Keywords: *Biodiversity management committees (BMCs), personal profile, biological diversity act*

Biodiversity Management Committees (BMCs) under local self-governments constitute the grass root level institutional mechanism to implement the provisions of National Biological Diversity Act, 2002, Rules (2004) and Access and Benefit Sharing (ABS) Guidelines, 2014, pertaining to biodiversity conservation, sustainable use, and distribution of benefits ensued from the use of bio-resources and related knowledge in a fair and equitable manner. The statutory obligations that come under their purview include documentation of bio-resources including indigenous animal and plant genetic resources of cultural and economic value and allied knowledge in the form of People's Biodiversity Registers (PBRs) and taking lead in promoting conservation and sustainable development of invaluable biodiversity falling within their jurisdiction. Moreover, BMCs are presumed to take part in ABS of bio-resources that can enhance the livelihood status of local communities.

Kerala has exemplary reputation of being the first state that established BMCs as well as Peoples Biodiversity Registers (PBR) in all Local Self-Government institutions (LSGIs). As per mandate, BMC is constituted with president of local body as chairperson, secretary of local body as secretary and a team of six nominated permanent residents in LSGI's jurisdiction, representing cross section of the society as members. Kerala State Biodiversity Board (KSBB) has put in place well-crafted guidelines to facilitate effective functioning of BMCs as per mandate. However, despite considerable headway made in such primary procedural formalities, much remains to be done for realizing the cherished organizational goals. This is especially true with regard to optimal and rational management of livestock genetic diversity that requires persistent efforts.

Though disaggregated data on pertinent personal attributes of members of BMCs could be a proactive tool in identifying and bridging gaps in local biodiversity governance, literature review reveals few studies in this regard. The study assumes significance in this context.

Materials and methods

The study was confined to two districts each, from northern, central, and southern zones of Kerala. The selection of districts was based on criteria viz. indigenous cattle population, documentary evidence on native livestock of conservation value and expert guidance by subject matter specialists (Anilkumar and Raghunandanan, 2005; Jayadevan *et al*., 2015; KSBB, 2016; NDDB, 2016; Singh, 2017; Anilkumar, 2018; Sreelakshmi and George, 2019). Accordingly, the districts of Kasargod and Kozhikode; Palakkad and Idukki; and Kottayam and Alappuzha were selected purposively from northern, central, and southern Kerala, respectively (Bryman, 2016). Five Biodiversity Management Committees (BMCs) from each selected district were chosen purposively based on key informant technique (Tremblay, 1957), wherein, key informants were the district level coordinators.

The organizational structure of BMC comprised of a chairperson, secretary and six other members including the convener. Data on personal profile of 240 respondents, eight from each BMC under study, were procured using structured questionnaire that was administered through e-surveys and telephonic interviews and subjected to frequency and percentage analysis.

Among the personal attributes studied, *Occupation* was operationally defined as the area/domain of work in which the respondents were engaged most of the time or derived income from, apart from their role as members of BMCs. *Training exposure* referred to the number of trainings undergone by respondents in the domain of biodiversity management. *Working experience* implied number of years of experience of the respondents as members or officials of BMCs. *Awards and recognitions received* referred to the number of awards or recognitions received by individual respondent for his or her performance in biodiversity related activities.

Results and discussion

It is obvious from the data (Figs.1 and 2) that representation of women as well as youth in the Biodiversity Management Committees under study was at a dismal low scale despite adherence to the norms of one third reservation for women, as mandated by the Biological Diversity Rules, 2004 (NBA, 2004). This implies that women's participation at grass root level biodiversity management machinery and decision-making processes remained marginal even with fifty percent reservation for women in local self- government institutions of the state, enviable social development indicators and plethora of gender-responsive missions for poverty alleviation and livelihood enhancement.

In the policy context, Kerala Gender Equality and Empowerment Policy, 2015 (Department of Social Justice- GoK, 2015) envisages participation of both genders in conservation activities and prevention of environmental degradation. The state Environment Policy, 2009 (Department of Environment- GoK, 2009) recommends women specific environment awareness programmes delivered through local bodies and women's organisations. However, it is high time to scrutinize the gender dimensions of our biodiversity policy towards synergies with Gender Plan of Action, 2015-20 of CBD (Secretariat of CBD, 2015). In its preamble, CBD recognizes the crucial role played by women in biodiversity conservation and its sustainable use and underlines the need for their active partaking in policy formulation and implementation at all levels. Proposed Post 2020 Biodiversity Framework (CBD, 2019) also envisages mainstreaming of gender perspective for accomplishment of Sustainable Development Goal (SDG) 5 on gender equality. Global biodiversity outlook 5, 2020 (Secretariat of CBD, 2020) recommends integration of gender dimensions into the national biodiversity management frameworks as a key strategy to address implementation challenges. Thus, owing to the widespread recognition of women's crucial role as users and managers of bio-resources, gender sensitive policies and programmes linked to biodiversity management and leadership in key institutions could be critical entry points to women empowerment. This would eventually facilitate accomplishment of objectives linked to biodiversity conservation and sustainable development as well.

A few reports have come out with findings on gender disaggregated data in biodiversity management spheres. For instance, Namibia, under its community based natural resources management programme, reported that women constituted 30 percent of conservation management committee members, engaged in indigenous plant genetic resources management (CBD Secretariat, 2017). An assessment report (UNEP/CBD, 2016) on national biodiversity management plans of CBD member countries indicated that 67% of plan reports had at least one reference to gender or women, while 33% did not talk about either. Women were mentioned as beneficiaries in 30% of reports, 28% mentioned women as stakeholders whereas, 19% characterised them as resource managers and only 1 % of reports referred to their role as agents of change. With respect to gender responsive outcomes, the sixth national report of India (MoEF&CC -GOI, 2018) has highlighted the representation and participation of women Self Help Groups in environment synced development programmes. However, inclusion of gender disaggregated data on leadership, managerial and decision-making roles at various levels of biodiversity governance could have brought more accountability.

Another notable finding is the minimal representation of youth in BMCs under study. Nearly half of the members were above fifty years old. At this juncture, it would be interesting to read this finding along with the demographic picture of the state that is aging faster in comparison with rest of India. The elderly population in the state is well above the national average, whereas the national demographics is in favour of youth (KSPB, 2020). While the rich experience of elderly work force could be a valuable human resource, there should be concerted efforts to ensure substantive inclusion of youth in decision making and political processes related to biodiversity management at grass root, national as well as

international levels. Global Youth Biodiversity Network of CBD (CBD, 2017) has been a role model that leverages perspectives of youth across nations to enhance achievement of Aichi Biodiversity Targets. The most recent observations from assessment of national reports indicate that among younger people, understanding of biodiversity is on rise at a rapid rate (Secretariat of CBD, 2020). As envisaged, youth could provide leadership in creating public awareness of the values of biodiversity and measures to conserve and use it sustainably. An empowered youth force could be instrumental in effective updating and implementation of National Biodiversity Strategy and Action Plan (NBSAP). They could also contribute towards innovative knowledge and technology base for scientific bioresources management. Moreover, our National Biodiversity Target no.1 pertains to capacity building with special focus on youth (MoEF&CC-GOI, 2014; Onial, *et al*., 2018). ENVIS Centre on Biodiversity (Fauna) under ministry of Environment, Forest and Climate Change has devised Green Skill Development Programme (GSDP) Mobile app to skill India's youth (MoEF&CC-GOI, 2019).

Another significant finding is about the prevalence of political/ social workers among BMC members (Fig. 3). The representation of stakeholder communities including farming, livestock and fisheries was minimal whereas, there was absolute absence of traditional knowledge holders and practitioners of indigenous medicine. This could be attributed to the political affiliation that might creep in while nominating members by the local bodies. However, as ecosystem management is a social process that addresses socio-economic and cultural diversity of local communities in addition to management of natural and genetic resources (UNEP-CBD, 2004), substantial inclusion of social and political workers could be justifiable to some extent. Nonetheless, there has been increasing evidence that an interdisciplinary approach could be the cornerstone for sustainable thoughts on eco-management (Bull *et al*, 2016). Indigenous wisdom and scientific acumen along with political will, could facilitate judicious decision making and implementation at grass root level administration and management of bioresources. Kerala Biological Diversity Rules, 2008 (Department of Environment, GoK, 2008) strategizes a multidisciplinary team in BMCs, representing a cross section of society comprising of crop, livestock and fishery farmers, practitioners of indigenous medicine, forest dwellers, tribal leaders, researchers, teachers, environmentalists, and other potential persons.

The ecosystem approach of biodiversity management that is envisaged in Convention on Biological Diversity (UN, 1992), strategic priority area on sustainable development of Global Plan of Action on Animal Genetic Resources (FAO, 2007) and World Summit on Sustainable Development (UN, 2002) cannot be materialized without interdisciplinary approach. Undoubtedly, documentation of availability and use of local bio-resources and chronicling of associated knowledge, innovations, applications, and practices held by local communities would necessitate an inter-disciplinary teamwork at grass root level biodiversity management institutions. Hence, it is imperative to integrate the ecosystem approach into multiple sectors and production systems impacting biodiversity such as agriculture, animal husbandry, forestry, fisheries and so forth and the same should reflect in institutional machinery for biodiversity management. UNEP-CBD guidelines on ecosystem approach for biodiversity management (UNEP-CBD, 2004) as well as Global Biodiversity Outlook 5 2020 (Secretariat of CBD, 2020) underlines the importance of increased stakeholder participation and inter-sectoral cooperation and communication at various levels including creations of networks for sharing experience and information.

The findings regarding respondents' educational status (figure 4) and experience in biodiversity management strike an optimistic note. However, deploying capacity building exercises towards optimal bio-literacy of the potential task force seems to be a strategic imperative. KSBB has instituted awards for model as well as for best BMCs (KSBB, 2020), though no awards have been set up for rewarding individual merits of members. (Figs. 6 and 7). The scheme for incentivizing BMCs

Personal Profile

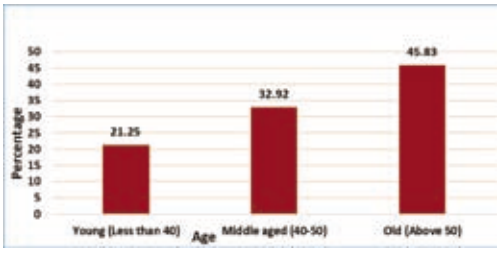

Fig. 1. Distribution of respondents based on age

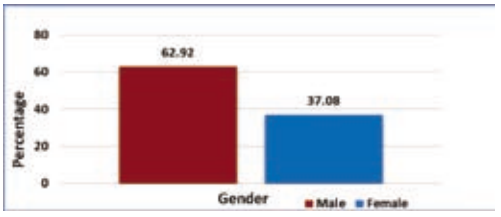

Fig. 2. Distribution of respondents based on Gender

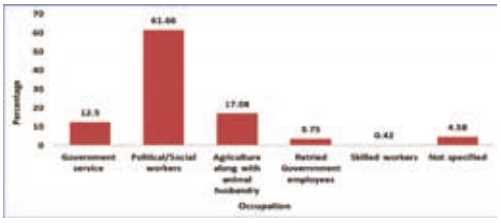

Fig. 3. Distribution of respondents based on Occupation

through criteria-based selection of model BMCs and their capacity building has been a well thought out strategy.

The findings regarding training exposure (Fig. 5) in biodiversity management among BMC members reveals that a vast majority received not more than one training. Only a few had attended two or three training programmes. This implies that there is absolute necessity for policy initiatives at organizational level for further capacity building of the members in biodiversity management realm. Lack of biodiversity education as well as ignorance of biodiversity related issues among stakeholders whose decisions and initiatives have an impact on biodiversity has been reported as an important factor hampering achievement of objectives mandated by Convention on Biological Diversity as well

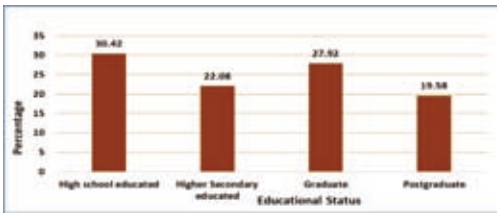

Fig. 4. Distribution of respondents based on Educational Status

Fig. 5. Distribution of respondents based on Training exposure in biodiversity management

All the respondents reported a working experience of 5 years in BMCs.

Awards/recognitions received by members

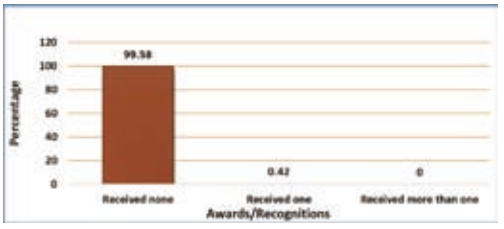

Fig. 6. Distribution of respondents based on Awards/recognitions received by members

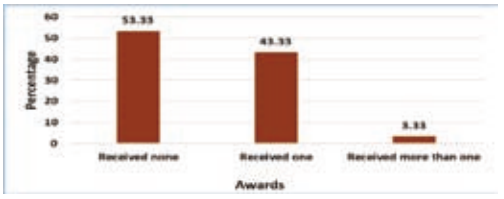

Fig. 7. Distribution of BMCs based on Awards received

as biodiversity erosion at an unprecedented rate (Navarro-perez and Tidball, 2012; Basnet *et al*., 2019). In this context, it would be pertinent to discuss about our National Biodiversity Target number one that mandates building biodiversity awareness including its value and measures for conservation and sustainable use among substantial section of

the population. This would essentially require institutional capacity building at various levels of governance. The Communication, Education and Public Awareness (CEPA) programme of CBD could be utilized as a strategic instrument to attain this target (Hesselink *et al*., 2007). A recent analysis of national reports of CBD member countries reveals that none of the Aichi Biodiversity Targets have been met fully within the stipulated period (Secretariat of CBD, 2020). Different educational, societal, technical, and institutional factors along with the lack of political will, dearth of empowered human resources and lack of public education and awareness have been recognized as obstacles in this regard (Navarro-Paraz and Tidball, 2012). Global Biodiversity Outlook 5, 2020 observes 'building human capital and institutions' (SDG 3, 4, 16) as a potential driver that contributes towards 'conservation and sustainable use' of biodiversity (Secretariat of CBD, 2020).

Conclusion

The study elucidates the need for gender dis aggregated data on leadership, managerial and decision-making roles at various levels of biodiversity governance along with a close scrutiny of the gender dimensions of our biodiversity management policy towards synergies with international instruments such as Gender Plan of Action of Convention on Biological Diversity (CBD). Also, there should be concerted efforts to ensure substantive inclusion of youth in decision making and political processes related to biodiversity management at grass root level. Integration with international platforms such as Global Youth Biodiversity Network of CBD and national platforms like Green Skill Development Programme of Ministry of Environment, Forest and Climate Change is the need of the hour. Another critical requirement would be the deployment of an interdisciplinary team of multiple stakeholders at grass root level Biodiversity Management Committees (BMCs), as this would facilitate ecosystem approach in biodiversity management. Also, the findings call for intense capacity building and incentivizing measures towards optimal bio-literacy of the potential task force at BMCs.

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Conflict of interest

The authors declare that they have no conflict of interests.

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Household waste reduction efficiency of *Hermetia illucens* larvae#

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Abstract

Waste management with limited resource input under minimum space is the need of the hour. Black Soldier Fly (BSF)/Hermetia illucens is a fly prevailing and flourishing in our tropical climatic condition and is a miracle insect since it can solve a multitude of global problems like waste management, animal feed production and energy production. This fly is highly reputed in converting low quality biomasses in to high protein, high energy larvae meal. In this study we are assessing the quantity of BSF larvae produced from food waste and the waste reduction efficiency of the BSF larvae. Four kilograms of food waste was kept in a modified bin with 6 replicates and the development of the larvae was through natural breeding. The study revealed that from the above said quantity, an average of 0.567kg of BSF larvae can be produced per bin and average waste reduction efficiency was 73.81%.

Keywords: *Black soldier fly, Black soldier fly larvae, food waste, natural breeding, waste reduction efficiency*

Roughly one-third of the edible parts of food produced for human consumption, gets lost or wasted globally, which is about 1.3 billion ton per year (Gustavsson *et al.,* 2011). Poorly managed waste serves as a breeding ground for disease vectors, contributes to global climate change through methane generation, and can even promote urban violence. Operating this essential municipal service requires integrated systems that are efficient, sustainable, and socially supported. Usually various techniques like composting, vermicomposting, biogas technology etc. are being adopted for the reduction of waste. The proper and economic disposal of slaughter house wastes is also a big

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problem and organic briquettes using biomass was found to be effective method. (Arun Sankar *et al.,*2022). Nowadays an emerging technology using black soldier fly (BSF) for the waste reduction has attracted the attention of many of the farmers because it differs from all other technology by the production of feed from waste. These insects can be reared on various kinds of organic wastes like vegetable wastes, fruit wastes municipal organic solid wastes (mixed waste of discarded fruits, vegetables, and food scraps produced by households, restaurants, markets, malls, companies, and public institutions), slaughter house waste, millings and brewery side, animal manures and even human faeces (human faeces from source separation toilets, faecal sludge from onsite sanitation technologies) (Lalander *et al.,* 2019). Adult BSF do not feed during their life (Rindhe *et al*., 2019), and is not considered as a pest or a vector. They do not carry pathogens and the larval activity significantly reduced *Escherichia coli* 0157:H7 and *Salmonella enterica* (Van Huis *et al.,* 2013). The annoyance of blood-sucking and biting flies can be reduced by use of illuminated fly traps. (Praveenkumar *et al.,* 2022). Besides, reduced emission of methane during waste decomposition (Dortmans *et al.,* 2017) and prevention of housefly breeding (Bradley and Sheppard, 1984) are the additional benefits. It takes only 18-20 days for the waste reduction. The major benefit along with the waste reduction is the yield of protein and fat rich BSF larvae and fertilizer as by-product. These larvae can be fed to poultry, fishes and pigs and thus can reduce the cost on feed, which constitute around 70-75 per cent of the total expenditure. The optimum energy and lysine levels in feed are important for economic production of meat production in Japanese quails. (Shibi Thomas *et al.,* 2021.)

Thus, these insects have the potential to provide promising solutions to two of modern agriculture's growing problems: the high cost of animal feed and the disposal of large amounts of waste (Givens *et al*., 2013). In the present study we are analysing the yield of the BSF larvae from food waste through natural breeding and waste reduction efficiency of BSF larvae in such natural system.

Materials and methods

The study was conducted at Eco farm, Department of Livestock Production Management, CVAS, Mannuthy. Six modified bins (Fig. 1) were taken for the study and few holes were made at the bottom of the plastic bin to remove the lechate from the bin and another bowl was placed below the bin to collect the lechate. A hole was placed on the lid of the bin and a T shaped PVC pipe was fitted in that hole, so that BSF (Fig. 2) can enter into the bin. One eggie made of cardboard piece was hung from the lid, and another eggie made of two tile pieces rubbered together were also placed in that bin. An arrangement for the larvae to crawl out of food when it reaches the prepupa stage was also made. Initially two kg of waste was put in each bin and all bins were placed in an open area under the shade of trees. The pungent smell of the waste attracted the BSF and they laid eggs on the eggies, near the corrugated surfaces of T shaped PVC pipes and also in the leafy portion of the vegetables in the waste. Eggs (Fig. 3) hatched within 4-5 days and larvae (Fig. 4) fell into the waste (the whole steps from the attraction of the flies till hatching of larvae are mentioned as natural breeding in this article). After 12 day of initial charging another two kg waste was put in each bin and the larvae were harvested on 18th day. Weight of the larvae and weight of the residual waste in each bin were measured. Thus, average quantity of larvae/ bin and average waste reduction efficiency was calculated. Weight of the residual food waste after treatment and waste reduction efficiency were calculated by the following formula:

Weight of the residual food waste after treatment = Weight of the bucket with larvae and waste after treatment - (weight of the bucket+ weight of the larvae)

$$\text{Substrate reduction (\%)} = \frac{\text{Total waste added - residual waste after treatment} \quad *100}{\text{Total waste added}}$$

Results and discussion

Weight of the bucket (A), weight of the bucket with larvae and waste (B), Quantity of larvae produced from each bin (C) weight of

Fig 1. Modified bin with food waste

Fig 2. Black Soldier Fly

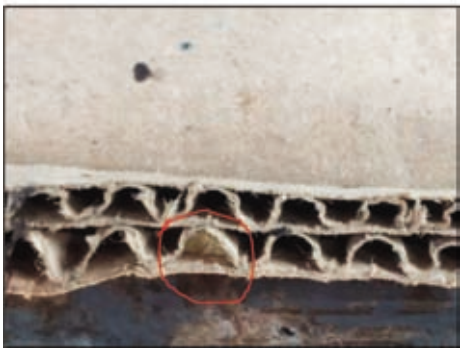

Fig 3. Egg of BSF

Fig 4. Black soldier fly larvae

residual waste after treatment (D) and waste reduction efficiency (E) are enlisted below (Table 1).

Average quantity of BSF larvae produced from each bin =

$$\frac{0.635+ 0.655+0.675+0.340+0.590+0.505}{6}$$

= 0.567 kg

Average waste reduction efficiency =73.81%

In the present study using a modified bin of 6 replicates and each containing four kg of food waste we could harvest an average of 0.567 kg of BSF larvae/bin. Gligorescu *et al*. (2020) used about 190 kg of food waste to produce 79 kg of BSF larvae by adding young BSF larvae, produced by artificial breeding of BSF to the waste. But in our study natural breeding was opted to allow the larvae to develop in the waste without artificial breeding, which might be the reason for lesser production. In the present study, the waste reduction efficiency was 73.81 per cent. Diener *et al*. (2011) studied the waste reduction and insect biomass produced with different types of wastes by the BSF larvae. It was found that 68 per cent waste reduction and a higher insect biomass yield with municipal organic waste followed by chicken manure (50 per cent reduction) and pig manure (39 per cent reduction). A greater waste reduction efficiency observed in the present study might be due to the difference in the content of the food waste and also due to the natural system of breeding adopted.

Conclusion

Present study revealed an average production of 0.567 kg of BSF larvae/bin from four kg of waste and their average waste reduction efficiency was 73.81 per cent. Though, a number of waste reduction methods do exist, BSF technology is an ideal one for waste reduction in home or in farm, as waste reduction is accompanied by the production of feed and fertilizer. Thus, BSF technology is a promising farmer friendly tool for waste management in animal farms with simultaneous production of a protein and fat rich larvae meal. As a developing country, it is high time that the potential of this fly be extensively utilized for extensive waste management strategies with emphasis on its pathogen inactivation efficiency.

Table 1. Parameters for assessing substrate reduction

	Bin 1	Bin 2	Bin 3	Bin 4	Bin 5	Bin 6
A (kg)	1.470	1.360	1.470	1.360	1.625	1.625
B (kg)	2.425	2.320	3.135	4.295	3.045	3.375
C (kg)	0.635	0.655	0.675	0.340	0.590	0.505
D (kg)	2.425-(1.470+.635) = 0.32	0.305	0.99	2.595	0.83	1.245
E (%)	92.00	92.375	75.25	35.12	79.25	68.87

Conflict of interest

The authors declare that they have no conflict of interest.

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Effect of supplementing limiting amino acids on growth performance and carcass traits of Gramasree male chicks fed with low protein diets#

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Abstract

An experiment was conducted to study the effect of reduced dietary crude protein (CP) diet supplemented with limiting amino acids on growth performance and carcass traits of Gramasree male birds up to eight weeks of age. A total of 280 day-old chicks were allotted randomly under five dietary treatments each with four replicates of 14 chicks under completely randomized design. The different dietary treatments viz., control diet (T1) was formulated as per ICAR (2013) nutrient requirements for Indian improved native birds with 21 per cent CP and 2800 kcal/kg Metabolizable energy (ME) using corn, soya bean meal, wheat bran and de-oiled rice bran. Treatment diets T2, T3, T4 and T5 were formulated by reducing CP content to 20, 19, 18, and 17 per cent, respectively with isocaloric value of 2800 kcal/kg ME and supplemented with methionine, lysine, threonine and tryptophan to meet the daily requirements. Weekly body weight and feed consumption were recorded. For the carcass study, two birds from each replicate were randomly selected and slaughtered humanely at eighth week of age. The results showed that the final body weight and cumulative weight gain were comparable among the treatments. The lowest cumulative feed intake and feed conversion ratio (FCR) were observed in 17 per cent CP containing treatment (T5), which was not significantly different from rest of the groups. The mean per cent breast meat yield, giblet yield, dressing yield and ready-to-cook yield were comparable among the five dietary treatments. The mean abdominal fat content in birds fed with 18 and 17 per cent (T4 and T5) were significantly ($p<0.05$) higher than the other treatment groups. The overall results indicated that the dietary CP of Gramasree male chicks can be lowered up to 17 per cent with the supplementation of methionine, lysine, threonine and tryptophan without affecting the growth performance of birds.

Keywords: *Low CP diet, limiting amino acids, Gramasree chicks, carcass trait*

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Poultry development in India has taken a quantum leap in the last four decades. Broiler meat production is growing in India at a rate of 12 per cent per annum (GOI, Ministry of Food Processing Industries, 2019). The demand for broiler meat is expected to grow stronger due to consumer preference, increasing income levels and changing food habits. But there are certain hurdles in the availability of quality protein source at adequate quantity and affordable prices which can adversely affect the future growth of poultry industry. Among the feed ingredients, soya bean meal and corn are the costlier ingredients which together constitute about 95 per cent of the total feed cost and are the major components in changing production and marketing scenario of poultry and poultry products (Megha *et al*., 2021). Along with feed cost, the increased concern over environmental pollution due to rising ammonia emission from poultry houses as an effect of feeding diets with high protein also pose a high risk to future expansion of the poultry sector. Therefore, to find out a suitable feeding regime that decrease the feed cost as much as possible without affecting the growth performance of birds satisfying the requirements of all major nutrients is the need of the hour.

Several nutritional strategies have been proposed to reduce the feed cost and to reduce the possible effect on environmental concern, one such strategy is reducing the crude protein (CP) content of the diet. If this strategy is to be effective and growth performance are not to be harmed, the level of limiting amino acids in such reduced CP diets must be maintained by supplementation. Practically, poultry ration based on corn-soya bean meal are deficit in methionine, lysine, threonine and tryptophan to meet the requirement of birds (Baker *et al*., 1993). Therefore, it became essential to supplement the synthetic feed grade form of these limiting amino acids in the diet to achieve the desired performance. Replacing the soya bean meal partially with the synthetic limiting amino acids having increased bioavailability and affordable price can reduce the total cost of production and improve the nutrient digestibility in birds without altering their growth performance. However, differences in crude protein level, amino acid fortification, feed ingredients, levels of amino acid requirement imposed and age of birds have reported to contribute to the discrepancies in the impacts (McGill, 2009).

Gramasree, a synthetic coloured dual purpose breed of chicken which was evolved from University Poultry and Duck Farm, Mannuthy in 2005, has become very popular due to its coloured plumage and brown shelled eggs similar to native chicken. Farmers in Kerala are showing interest in rearing Gramasree male chicks due to the low chick price, coloured plumage and high selling price compared to commercial broiler chicken. Considering the importance of rearing Gramasree cockerels for meat purpose and wide acceptability of this breed among farmers, studies have been conducted at Kerala Veterinary and Animal Sciences University to standardise the nutrient requirements of Gramasree cockerels at different phases till marketing. Keeping in view of the importance of profitable rearing of Gramasree birds for meat and reducing the nitrogen excretion, the present experiment was carried out study the effect of reduced dietary crude protein supplemented with methionine, lysine, threonine and tryptophan on growth and carcass characteristics of Gramasree birds.

Materials and methods

For conducting the trial, 280 day-old Gramasree male chicks were procured from University Poultry and Duck Farm, Mannuthy. All the chicks were wing banded and randomly allotted to five dietary treatment groups each with four replicates of 14 chicks in a completely randomized design and housed in deep litter system up to eight weeks. Uniform management practices such as scientific feeding, watering, lighting and vaccination were followed for all the groups throughout the experimental period.

Experimental rations

The experimental birds were fed with a control diet (T1) formulated with 21 per cent CP and 2800 kcal/kg ME according to ICAR (2013) nutrient specifications for Indian improved native birds and their crosses using corn, soya bean meal, wheat bran and de-oiled rice bran (DORB) to meet out the requirements

of first four limiting amino acids *viz*. methionine, lysine, threonine and tryptophan at the levels of 0.46, 1.10, 0.70 and 0.20 per cent, respectively by using synthetic amino acids. The other treatment diets (T2 to T5) were formulated isocaloric as that of control by reducing CP content to 20, 19, 18, and 17 per cent, respectively by balancing the amino acid levels as that of control using synthetic source. The same feed composition was followed throughout the experimental period.

Corn, soya bean meal, DORB and wheat bran were analysed for the first four limiting amino acids *i. e.* methionine, lysine, threonine and tryptophan by liquid chromatography and the results are presented in Table 1. The ingredient composition of the ration fed to experimental birds are presented in Table 2. The feed samples were subjected to proximate analysis as per AOAC (2005) which is presented in Table 3. The body weight and feed consumption of individual birds were recorded at weekly intervals from day-old to eight weeks of age and weekly body weight gain and feed conversion ratio were calculated from the data obtained. The cost of feed per bird and per kilogram of body weight of bird were calculated using the cumulative feed consumption and final body weight of the experimental birds. At the end of the experiment, two birds from each replicate were randomly selected and slaughtered humanely to study the carcass traits. Data on breast meat yield, giblet yield, abdominal fat yield, dressing per cent and ready to cook yield were estimated and expressed as per cent of live body weight.

Result and discussion

Body weight

The final body weight, cumulative body weight gain, feed consumption and FCR at eighth week of age are presented in Table 4. The cumulative body weight and weight gain of birds at eighth weeks were not significantly different between the treatments.

The present finding is in agreement with Shao *et al*. (2018) and Joseph *et al*. (2018), who assessed that reduction in dietary crude protein of birds with supplementation of essential amino acids did not affect the final body weight and weight gain. The results are also in par with the findings by Divya (2014) who experimented on Gramasree cockerels with dietary treatments having 2800 kcal/kg ME and CP ranging from 22 to 18 per cent without limiting amino acids supplementation. Contrary to the findings of the present study, Namroud *et.al.* (2008) observed that reduction below two per cent in protein level even with amino acid supplementation exhibited significant decrease in weight gain of broilers.

From the results of the present study, it is evident that, the limiting amino acid levels in low CP diets were so near to the true requirement of the birds, thus optimum to support their growth as that of control diet. If the level of limiting amino acids in low CP diets was inadequate, then it would have caused a deficiency and therefore growth depression (McGill, 2009). If excess level of limiting amino acids were present in diet, it would have catabolised to uric acid with the expenditure of 2-14 ATP from the stored energy (Costa *et al*., 2001).

Feed consumption

At eighth week, the cumulative feed consumption and mean cumulative FCR of birds were not significantly affected by dietary treatments. This result is in close agreement with the findings of Shao *et al*. (2018), Joseph *et al*. (2018) and Van Harn *et al.* (2019), who had also shown that reducing the dietary CP along with essential amino acid supplementation

Table 1. Amino acid contents in feed ingredients used for the trial (mg/kg)

Amino Acids	Corn	Soya bean meal	De-oiled rice bran	Wheat bran
Lysine	24.99	320.50	45.07	212.01
Methionine	18.62	103.64	26.01	19.06
Threonine	11.69	65.33	127.52	37.99
Tryptophan	96.65	75.95	Nil	21.04

Table 2. Ingredient composition of ration used in the trial

Ingredients	Per cent composition				
	T1	T2	T3	T4	T5
Corn	58.90	59.70	60.60	61.92	62.10
DORB	1.40	3.95	5.00	7.00	10.00
Soya bean meal	34.90	31.65	28.53	25.00	21.30
Wheat bran	1.55	1.40	2.40	2.50	2.80
Dicalcium phosphate	1.80	1.80	1.80	1.80	1.80
Calcite	1.30	1.30	1.30	1.30	1.30
Salt	0.15	0.15	0.15	0.15	0.15
Limiting amino acids (g/100kg feed)					
Methionine	0	10	40	70	100
Lysine	0	0	25	120	225
Threonine	0	40	80	120	170
Tryptophan	0	0	0	20	40
Feed additives (g/100kg feed)					
Vitamin AB_2D_3K mix[1]	25	25	25	25	25
Toxin binder[2]	75	75	75	75	75
Anticoccidial[3]	25	25	25	25	25
Choline chloride 60 %[4]	150	150	150	150	150
Trace mineral mixture[5]	100	100	100	100	100
Liver tonic[6]	30	30	30	30	30
Probiotic[7]	30	30	30	30	30

[1]Provimi (A+B2+D3+K): Vitamin Premix, contents per g- vitamin A -82,500 IU, vitamin D3- 12000 IU, Vitamin B2–50 mg, vitamin K- 10mg, vitamin B1 -4.0 mg, vitamin B6 -8.0 mg, vitamin B12 -40 mcg, Niacin -60 mg, calcium panthothenate -40 mg and Vitamin E -40 mg (Cargill animal nutrition Pvt.Ltd.)

[2]UTPP: Toxin binder a powerful blend of Hydrated Sodium Aliminosilicate, Organic Acids, Activated Charcoal and Natural Herbal Ingredients (NEOSPARK Drugs and Chemicals Pvt. Ltd)

[3]Nimax: Granular. Composition- Premix containing a combination of maduramicin 1.5% and nicarbazin 16 % (HUVEPHARMA)

[4]Choline chloride 60 per cent. Contents: each Kg of choline chloride dry 60 % powder contains a minimum 600 g of choline chloride (ANICHOL-60)

[5]ULTRA-TM: Each 5kg contains-Manganese 270 g, Zinc 260 g, Iron 100 g, Copper 10 g, Iodine 10 g, Selenium 1.5 g (NEOSPARK Drugs and Chemicals Private Ltd.).

[6]Liv.52 protec: Production enhancer, hepatic stimulant, growth promoter, liver tonic powder (The Himalaya Drug Company)

[7]Alvizyme plus: Contain digestive enzymes, probiotics and yeast. (Alembic Ltd.)

Table 3. Analysed per cent chemical composition of experimental ration (On dry matter basis)

Parameter	Experimental Diet (Per cent)				
	T1	T2	T3	T4	T5
Dry matter	90.60	91.18	90.82	90.03	90.55
Crude protein	21.33	20.57	19.29	17.93	17.11
Crude fibre	3.45	4.26	4.09	4.62	5.21
Ether extract	2.78	2.82	2.78	2.80	2.78
Total ash	8.20	7.94	6.95	7.56	7.54
Acid insoluble ash	1.40	1.32	1.30	1.22	1.35
Calcium	1.16	1.11	1.18	1.14	1.10
Phosphorus	0.69	0.74	0.70	0.62	0.71
ME (kcal/kg) *	2811	2824	2818	2837	2845

*Calculated

Table 4. Effect of different dietary treatments on body weight, feed consumption and carcass traits of Gramasree male chicks up to eight weeks of age

Parameters	Treatment Groups (Dietary CP Levels)						
	T1 (21%) (Control)	**T2** (20%)	**T3** (19%)	**T4** (18%)	**T5** (17%)	**F** value	**p** value
Body weight at 8th week (g)	836.14 ±7.35	812.53 ±25.15	802.32 ±27.19	809.79 ±16.98	836.85 ±18.89	0.61^{ns}	0.66
Cumulative body weight gain up to 8th week (g)	801.44 ±12.10	777.68 ±10.33	767.82 ±16.11	775.28 ±8.84	802.42 ±25.15	0.31^{ns}	0.87
Cumulative feed consumption up to 8th week (g)	2567.8 ±4.87	2520.77 ±51.89	2558.89 ±36.67	2555.66 ±10.94	2437.79 ±34.73	2.67^{ns}	0.07
Cumulative FCR up to 8th week	3.08 ±0.02	3.16 ±0.08	3.19 ±0.08	3.15 ±0.08	2.91 ±0.07	2.86^{ns}	0.06
Pre slaughter body weight (g)	914.50 ±28.32	888.75 ±22.80	896.25 ±22.33	850.00 ±17.89	878.00 ±25.53	0.61^{ns}	0.56
Breast meat (%)	8.31 ±0.31	8.31 ±0.35	8.11 ±0.17	7.50 ±0.24	7.62 ±0.32	1.95^{ns}	0.12
Abdominal fat (%)	0.09^{a} ±0.02	0.16^{a} ±0.04	0.11^{a} ±0.03	0.29^{b} ±0.15	0.63^{b} ±0.05	1.18*	0.04
Giblet weight (%)	4.87 ±0.06	4.90 ±0.10	4.96 ±0.18	5.05 ±0.21	5.06 ±0.32	0.15^{ns}	0.96
Dressing yield (%)	63.51 ±5.05	68.50 ±2.18	68.32 ±1.11	69.73 ±1.01	65.09 ±3.69	1.13^{ns}	0.36
Ready to cook yield (%)	68.38 ±0.52	73.40 ±0.32	73.34 ±0.71	74.78 ±0.86	70.15 ±1.95	0.69^{ns}	0.60

Mean values bearing same superscript within a row do not differ significantly
ns-non significant
*significant ($p<0.05$)

had no effect on feed consumption. But in contrast to these references, Bregendahl *et al*. (2002) after experimenting on broilers with diet having CP reduced from 23 to 18.5 per cent, with amino acid supplementation reported that feed consumption was lower in low CP diet groups. Shao *et al*. (2018) reduced the CP level of broiler diet up to 17 per cent by balancing with amino acid supply whereas Joseph *et al*. (2018) experimented on Swarnadhara chicks by reducing CP up to 19 per cent with limiting amino acid supplementation. Similar to the present study, both of them observed no significant difference among the cumulative FCR among the treatments. On the contrary, the eighth week mean cumulative feed consumption of Gramasree cockerels obtained by Divya (2014) by feeding the experimental birds with diets containing 22, 20 and 18 per cent CP levels (2800 kcal/kg ME) without balancing the limiting amino acids levels was lower when compared to the present study. From the present study, it is evident that, reducing the dietary crude protein level with limiting amino acid supplementation up to 17 per cent in the diet of Gramasree male chicks did not affect the feed consumption and feed efficiency of birds.

Carcass traits

Statistical analysis of the data pertaining to the carcass characteristics studied did not show any significant difference between groups except for the abdominal fat content (Table 4). The mean abdominal fat content in 18 and 17 per cent CP fed groups (T4 and T5) were significantly ($p<0.05$) higher than that of other treatment groups.

Raju *et al*. (1999), Kamran *et al.* (2004) and Namroud *et al*. (2008) also agrees

with the insignificant variation of mean dressed weight, giblet yield, breast meat yield and dressing per cent among low CP diets after balancing with amino acid supplementation. Divya (2014) who reduced the dietary CP level of Gramasree cockerels from 22 to 18 per cent without limiting amino acid supplementation also observed a non-significant variation in carcass characteristics. The increased abdominal fat yield of birds fed with low CP diets observed in this study was experienced by many researchers previously. Raju *et al*. (1999), Joseph *et al*. (2018) and Karthika *et al*. (2019) have also reported that low protein diets with amino acid supply in the diets increased the abdominal fat of the broilers.

A reduction in CP level causes an increase in ME:CP ratio. In the present study, the ratio was 133:1 in control diet which increased to 165:1 in 17 per cent CP containing diet. A greater ME:CP ratio of low CP diets increases the fat deposition in the carcass. An increased CP content in diets increases the heat increment by deamination and transamination of surplus amino acids to other metabolites in the body. Meanwhile in low CP diets balanced with amino acid supply, energy expenditure in the form of heat of digestion is saved and is added to the body fat reserve (Bartov *et al*., 1974).

Conclusion

Based on the overall results of this study, it could be concluded that, the dietary crude protein level of Gramasree male chicks can be reduced up to 17 per cent with limiting amino acids supplementation to reduce the feed cost without affecting the growth performance of birds for rearing up to eight weeks of age.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Molecular detection of methicillin resistant *Staphylococcus aureus* associated with mastitis in goats

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Abstract

Antimicrobial resistance is an emerging concern in the world while combating with a wide range of pathogen associated with various disease conditions. It is considered as a silent pandemic nowadays. A constant monitoring and measures for controlling emergence of antimicrobial resistance are crucial in the current situation. The present study was conducted with the objective of detection of methicillin resistant Staphylococcus aureus (MRSA) associated with mastitis in goats. Milk samples were collected from 66 goats suffering from mastitis and screened for the presence of S. aureus. Conventional microbiological tests and species specific PCR were employed for the species confirmation. A total of 10 samples (15.15%) were found to be positive for S. aurues. Among the ten isolates of S. aureus obtained two isolates (3.03%) were found to harbour mecA gene suggestive of the presence of MRSA phenotype. All the isolates were negative for the presence of pvl gene which is present in highly virulent strains. In short, even though the study demonstrated the presence of MRSA at a lower rate, the results are of a marked public health significance due to the zoonotic significance of the pathogen.

Keywords: *Mastitis, caprine, MRSA, PCR*

Staphylococcus aureus is an opportunistic pathogen of human and other mammals which has increasing clinical and veterinary importance as it has capability to develop antimicrobial resistance rapidly (Foster, 1996). The injudicious use of antibiotics has led to the emergence of antibiotic resistant *S. aureus* strains, most significant being methicillin resistant *S. aureus*. Methicillin resistant *S. aureus* (MRSA) is an emerging zoonotic pathogen of clinical significance worldwide. It can lead to several infections that are difficult to treat due to multidrug resistance. Methicillin is a beta lactam antibiotic that inhibits penicillin binding proteins that are involved in the synthesis of cell wall component of bacterium, peptidoglycan (Stapleton and Taylor, 2002). The methicillin

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resistance emerges due to the expression resistance emerges due to the expression of penicillin-binding protein (PBP2a), a foreign PBP that can resist the action of methicillin at the same time perform function of host PBPs (Kitti *et al*., 2011). The emergence of MRSA is a public health threat of utmost importance. The emergence of antimicrobial resistance is considered as a silent pandemic. (Mahoney *et al.,* 2021). Panton- valentine leukocidin is a beta pore forming cytotoxin is related with tissue necrosis and also causes disruption of leukocyte membranes. *Staphylococcus aureus* strains carrying PVL are highly virulent and rapidly transmissible strains than PVL negative *S. aureus* (Karmakar *et al*., 2018). The presence of this virulent factor in *S. aureus* is important in pathogenicity of bacteria.

Mastitis is an inflammation of the mammary gland and is the most debilitating disease in dairy goats which demands huge economic investment in treatment (Marogna *et al.,* 2010).

In developing countries goats are remarkable for the livelihood of the large population especially to disadvantaged sections of society who are prone to penury and undernourishment. According to the National Livestock census (2019), the total goat population in India was 148.88 million which forms about 27.8 per cent of the total livestock population. Milch animals constitute 69.65 per cent of the total goat population and this is the population susceptible to mastitis. The occurrence of MRSA in goats has to be constantly monitored as it can lead to serious public health issues. Hence the present study has undertaken to assess the prevalence of MRSA in caprine mastitis.

Materials and methods

Sixty-six goats suffering from clinical mastitis that had been presented at University Veterinary Hospitals (UVH), Mannuthy and Kokkalai during the period from April 2019 to March 2021 formed the subjects of the study**.** Milk samples were collected from the udder halves with mastitis after taking all aseptic precautions. Primary isolation of bacteria was performed in Brain Heart infusion agar (BHIA; Himedia, M211) by directly streaking on to agar and incubating at 37℃ for 24 hr. The isolates obtained were identified using the colony morphology gram staining and biochemical tests as per Barrow and Feltham (1993). Deoxyribonucleic acid was isolated from the cultures of bacteria using snap chill method. Polymerase chain reaction was performed to confirm the *S. aureus* isolates using species specific primers that target the *23S rRNA* gene of *S. aureus* (El-Razik *et al*., 2010). The presence of *mec*A and *PVL* in *S. aureus* isolates were detected by PCR using specific primers as per Kobayashi *et al.* (1994) and Pajic *et al.* (2014), respectively (Table 1.).

The PCR reaction mix was made of 5 µL of template DNA, 12.5 µL of PCR master mix (2X PCR Smart mix, Takara, Japan) forward and reverse primer of 100nM/ µL concentration 1µL (Sigma Aldrich), sterile nuclease free water 5.5µL and the total volume was made to 25µL. The amplification procedure followed for 23SrRNA was an initial denaturation at 94℃ for 5 min followed by 30 cycles of denaturation (94℃ for 45 s), annealing (55.8℃ for 45 s), extension (72℃ for 90 s), followed by final extension at 72℃ for 10 min. The amplification procedure followed for *mecA* gene was an initial denaturation (95℃ for 3 min) followed by 30 cycles of denaturation (95℃ for 45 s), annealing (60℃ for 60s), extension (72℃ for 60 seconds) and a final extension at 72℃ for 7 min. The amplification procedure followed for *pvl* gene was an initial denaturation (95℃ for 5 min) followed by 30 cycles of denaturation (95℃ for 30 s), annealing (59℃ for 30s), extension (72℃ for 60s) and a final extension at 72℃ for 5 min). The DNA isolated from known *S. aureus* constituted the positive control (Tresamol *et al*., 2018) and DNA from known *Escherichia coli* constituted the negative control (confirmed at Department of Veterinary Epidemiology and Preventive Medicine, College of Veterinary and Animal Sciences, Mannuthy). The PCR products were subjected to 1.5 per cent agarose gel electrophoresis for visualisation and documentation of the amplified products.

Results and discussion

Milk samples from all the 66 lactating goats with clinical mastitis yielded growth on

primary isolation on brain heart infusion agar. Gram's staining revealed that 80.31 per cent of the cases had (53/66) gram positive cocci and 19.69% (13/66) isolates were gram negative bacilli. The colonies of gram positive cocci were subjected to catalase and coagulase test to identify *S. aureus* and this was confirmed by sub culturing on mannitol salt agar. Based on colony morphology, Gram's staining and biochemical tests, 10 isolates were tentatively identified as *S. aureus*. The isolates obtained were further confirmed by species-specific PCR targeting 23S *rRNA* (Hunt *et al*., 2006), in which all the isolates yielded amplicons of 1318 bp confirming species identification (Fig.1.). Similar to our work, Yamagishi *et al*. (2007), El-Razik, *et al*. (2010) and Tresamol *et al*. (2018) used the same PCR technique for species level identification of *S. aureus* from bubaline and bovine milk respectively. In short, the prevalence of caprine mastitis was observed to be 15.15 per cent during a period of 8 months in the present study.

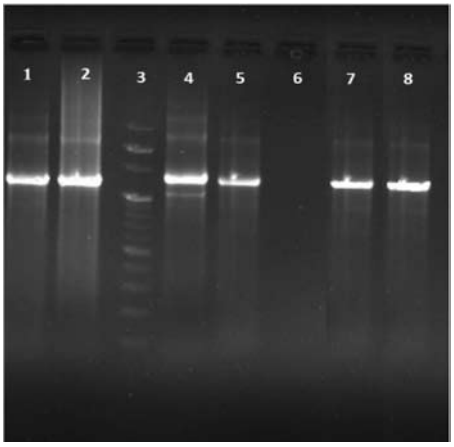

Fig. 1. Agarose gel electrophoresis of *23S rRNA* specific PCR of *S. aureus* (Lane 1: Positive control, Lane 6:Negative control Lane 2,4,5,7,8: Positive samples (1318 bp),Lane 3:DNA Marker (100 bp)

Fig. 2. Agarose gel electrophoresis of *mecA* specific PCR of *S. aureus* (Lane 6: DNA Marker (100 bp), Lane 4 and 5: Positive samples (303 bp), Lane 7: Negative control Lane 8: Positive control

A variable prevalence rate of *S. aureus* has been reported in lactating animals by different authors. Kini *et al.* (2019) reported a 51 per cent occurrence of *S. aureus* in goat mastitis from Wayanad, Kerala. Danmallam and Pimenov (2019) reported a 20 per cent prevalence of *S. aureus* in caprine mastitis from Moscow, Russia. Mugabe *et al.* (2017) reported an 83 per cent prevalence in goat mastitis from Botswana, Pakistan. Islam *et al.* (2011) reported *S. aureus* (36.36%) as the major pathogen associated with caprine mastitis from Mymensingh, Bangladesh. Najeeb *et al*. (2013) reported a 61.64 per cent *S. aureus* prevalence in caprine mastitis. Jose *et al.* (2021) reported 32.84 per cent occurrence of *S. aureus* in bovine mastitis from Thrissur, Kerala. Low prevalence of 23 per cent and 18.18 per cent were reported by Kumar *et al.* (2016) and Nabih *et al.* (2018) from Pakistan and Egypt, respectively.

Ten isolates yielded amplicons of size 1318bp confirming *S. aureus*. These ten isolates were subjected to PCR targeting *mecA* gene and *pvl* gene. Only 2 isolates yielded amplicons of size 303bp whereas none yielded amplicons for *pvl* gene. Detection of methicillin resistance in staphylococci is a tedious process in the clinical microbiology laboratory due to the heterogeneity of the bacterium under test. The detection of resistance in these isolates has been disturbed due to variability in the standard techniques used in determining resistance to methicillin. Resistance to methicillin is due to *mec*A gene which is part of a mobile genetic element called the staphylococcal cassette chromosomes (SCC) *mec* (Wu *et al*., 1996), and it represents a marker of methicillin resistance when molecular techniques such as PCR are used for screening the methicillin resistant gene in *S. aureus.* In the present study methicillin resistance was detected by using

PCR targeting *mec*A gene. All the 10 species confirmed isolates were when subjected to PCR for detection of presence of *mec*A gene, only two yielded *mec*A gene amplicons (Fig. 2.). Accordingly, the prevalence of MRSA in caprine mastitis cases was calculated as 3.03 per cent. Similarly, Aras *et al*. (2012) reported a low prevalence of 4.76 per cent in caprine mastitis cases reported in Turkey. The prevalence of *mec*A gene reported by Obaidat *et al*. (2018) and Altaf *et al.* (2020) in *S. aureus* isolates from goat milk were 11.5 and 6.55 per cents, respectively. In contrast, a higher prevalence of 50 per cent was reported by Suchithra *et al*. (2015) in *S. aureus* isolates from caprine clinical mastitis cases of Thrissur, Kerala whereas Persson *et al.* (2021) could not detect *mec*A gene in milk samples and nasal swab of goats.

High prevalence of MRSA had been detected in bovine mastitis (Kumar *et al*., 2011., Jisha *et al*., 2020.). The reason for the variation in occurrence between the species can be due to multiple causes such as the use of antibiotics, hygiene practices, and disease control programmes which influences the occurrence. The group under study constituted only a small proportion of a large population which might be the reason for the low percentage of detection of MRSA in the present study. None of the isolates yielded amplicons for *pvl* when subjected to PCR specific for *pvl* gene. This result was in line with the findings of Tegegne *et al.* (2019) where no *S. aureus* from goat mastitis samples were reported to harbour the *pvl* gene. The presence of MRSA in goats even at a low rate is of great public health significance. Hygienic precautions to control and constantly monitor the scene for the presence of methicillin resistance are very important as this pathogen is zoonotic and can lead to a pandemic if left unnoticed.

Conclusion

Antimicrobial resistance is an emerging issue in treating infectious diseases. It can be considered as a silent threat that could reverse the entire development that has been achieved in the field of treating infectious diseases. Constant monitoring and control measures are equally essential in controlling the emergence of antimicrobial resistance**.**

Acknowledgement

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Conflicts of interest

The authors have no conflicts of interest to declare.

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Astrocytic reaction in furious and paralytic forms of rabies with reference to GFAP expression in dog brain samples positive for rabies*

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Abstract

The aim of this study was to evaluate the astrocytic reaction in infected brains of dogs in that had been afflicted with the furious and paralytic form of rabies. Brain samples from 23 dogs that were positive for rabies were collected along with six brain samples that were negative for rabies. GFAP (Glial fibrillary acidic protein), specific astrocytic marker was used to differentially analyse the immunoreactivity of astrocytes to GFAP in specific brain regions in two the clinical forms of rabies viz, the furious and paralytic forms. The study showed the extent of astrogliosis in specific regions of brain in the two forms of rabies and also the intensity of GFAP expression. Region wise astrogliosis, was more diffuse in the brains of animals afflicted with the furious form of rabies but were more localized in the brains of animals with the paralytic form, specifically into the white matter of cerebellum.

Keywords: *Rabies, Astrocytes, GFAP*

Glial cells provide structural and functional support to the neuronal cells and account for 90 percent of the whole cells of CNS. Astrocytes are an integral component of the glial cell society in the CNS. They are multipolar, star shaped cells and are morphologically classified into protoplasmic cells observed in grey matter of the brain and fibrillary cells present in the white matter of the brain (Brat, 2018). Astrocytes are activated at any sort of CNS insult, either trauma or infection and respond to such situations through hypertrophy and/or hyperplasia. GFAP are the intermediate filament proteins that are commonly present in astrocytes acting as a specific marker of these cells and are helpful in delineating the pathological response of astrocytes at each stage of disease progression.

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Viral infections affecting the CNS are numerous and among these, the most important reviewed and researched one is rabies infection (Dhaka P and Vijay D, 2018). Both in humans and animals, the disease possesses a dreadful course with a fatal end (Krithiga *et al*., 2019). Though the disease was known from time immemorial, the actual pathogenesis through which the disease attains its furious or paralytic form is still a mystery to scientific society. Time demands an appropriate measure to differentially analyse the roles played by each cell of the CNS, cellular proteins like cytokines and the immune mediatory cells in the pathogenesis and course progression of the disease. This study intends to look into the astrocytic response in accordance with the infection and variation in the response of the same in two different forms of rabies. Immunohistochemistry using GFAP and its expression in brain tissue is considered as a reliable parameter to evaluate the extent of astrocytic activation/response to the disease.

Materials and methods

The animal anamnesis was collected from the animal owner when the carcass suspected for rabies was brought for post-mortem examination. For diagnosis of rabies in suspected cases, the brain impression smear was carried out using d-FAT. Cases positive for rabies under study were classified into two forms based on the clinical signs exhibited by the animal (Tepsumethanon *et al*., 2016). Brain samples collected were sliced based on the region required for the study and preserved in 10 per cent NBF.

Immunohistochemical analysis for Glial Fibrillary Acidic Protein positive astrocytes in the intended regions (*viz,* cerebrum, cerebellum, hippocampus and brain stem) of brain samples positive for rabies and brain samples negative for rabies, that served as the control, (5µm thickness) were carried out using GFAP as the primary antibody (Vector Laboratories). The IHC procedure was performed using Super Sensitive TM Polymer-HRP IHC Detection Kit (QD420- YIKE) from Biogenex. Antigen retrieval solution used for the IHC was 0.1M Sodium citrate buffer (pH 6.0).

Formalin fixed paraffin embedded brain tissues were sectioned at 5µm thickness, collected on APES coated slides and heat fixed. The sections were deparaffinized by immersing the slides in xylene and then hydrated through descending grades of alcohol and finally treated in distilled water. Antigen retrieval was carried out by immersing these slides in sodium citrate buffer and incubating at 95^{0}C for 20 minutes. After this, the sections were acted upon by the peroxide block and power block solutions for 3 minutes each. The sections were then incubated with 50µl of primary antibody at 4^{0}C overnight. Later these sections are incubated with enhancing reagent available in the kit and then followed by the secondary antibody. After a through wash, DAB solution was added to the sections and incubated for 20 minutes. The sections were counterstained with Ehrlich's haematoxylin and treated with tap water (blueing) at room temperature. Slides were then treated with increasing grades of alcohol (50 per cent to 100 per cent) and cleared using xylene and mounted with cover slips using DPX mountant.

Immunohistochemical scoring

Grey matter and white matter of all the four regions of brain were examined under 40X magnification (high power) to evaluate the intensity of immunostaining and number of positive cells. Based on the intensity of staining, scores were assigned. The number of GFAP positive astrocytes per unit area was assessed by calculating the mean number of astrocytes counted in five random fields examined under high power magnification. Only the astrocytes with brown coloured cytoplasm and processes were considered GFAP positive (Machado and Alessi, 1997).

Statistical analysis

IBM SPSS Statistics software, version 21.0 was used for statistical analysis. One-way ANOVA was carried out followed by Duncan Multiple Range test (DMRT) for pair wise comparison in analysing the immunoreactivity for GFAP.

Results and discussion

In the present study samples of the

brain of dog carcasses with a clinical history of neurological abnormalities were screened for rabies using d-FAT. A total of 23 brain samples that were positive for rabies and 06 samples (control group) that were negative were collected for the study.

Immunoreactivity of GFAP in two forms of rabies

The semi quantitative analysis of GFAP expression was carried out in two forms of rabies and immunoreactivity in different regions in furious and paralytic forms was recorded respectively (Fig. 1 and 2).

The intensity of immunoreactivity to GFAP was scored and differentially analysed in the two forms of rabies as shown in Table 1. In both forms of rabies, an intense expression of GFAP protein (63 per cent of paralytic cases and 75 per cent of furious form) was observed.

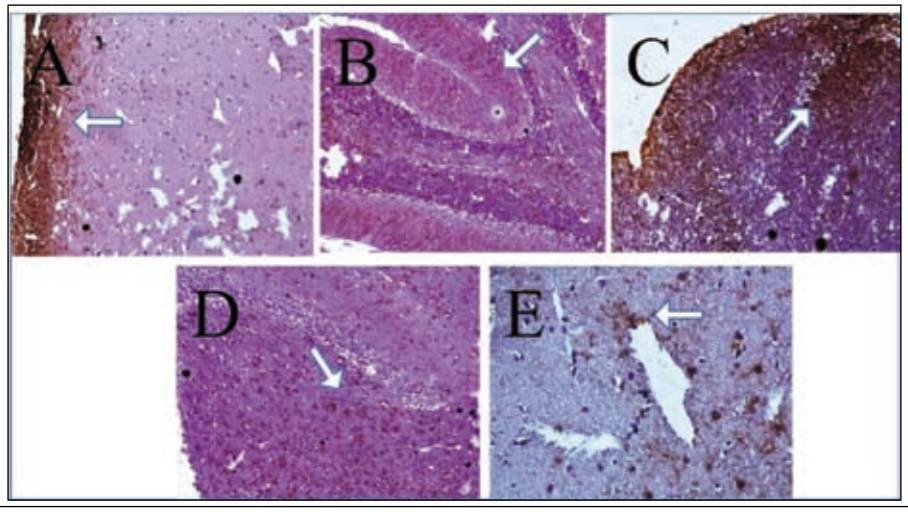

Fig. 1. GFAP reactivity in furious form of rabies (100X; DAB stained IHC sections counter stained by Heamatoxylin) A: Cerebrum- hypertrophy of astrocytes in glial limitans; B: Cerebellum- Astogliosis of white matter and extension of processes to the grey matter; C: Brainstem- astrogliosis; D: Hippocampus- astrocytosis near the dentate gyrus and E: Around the blood vessels in hippocampus(400X)

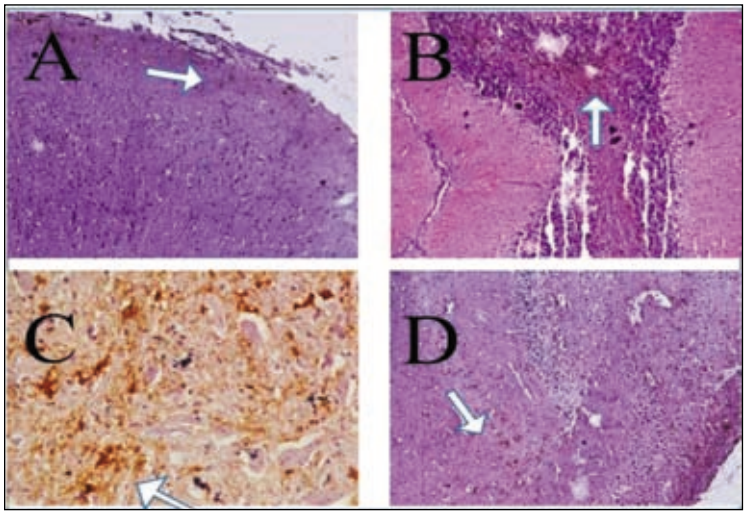

Fig. 2. GFAP reactivity in paralytic form of rabies (DAB stained IHC sections counter stained by Heamatoxylin) A: Cerebrum- mild hypertrophy of astrocytes in glial limitans (100X); B: Cerebellum- Moderated astogliosis of white matter and mild extension of processes to the grey matter (100X); C: Moderate astrogliosis of hippocampus (400X); D: Astrogliosis in the Brain stem (100X).

Table 1. Classification of cases according to GFAP reactivity

Intensity Score	Control		Paralytic Rabies Group		Furious Rabies Group		Overall	
	No.	Per cent	No.	Per cent	No.	Per cent	No.	Per cent
+	3	50.0	0	0.0	1	8.3	4	13.8
++	3	50.0	4	36.4	2	16.7	9	31.0
+++	0	0.0	4	**36.4**	5	**41.7**	9	31.0
++++	0	0.0	3	**27.3**	4	**33.3**	7	24.2
Total	6	100.0	11	100.0	12	100.0	29	100.0

Table 2. Comparison of No. of positive cells per unit area in GFAP expression between groups

Period	CONTROL	PARALYTIC	FURIOUS	F-value (P-value)
Cerebrum GM	2.08 ± 0.33	2.27 ± 0.26	2.75 ± 0.26	1.471^{ns} (0.248)
Cerebrum WM	6.33 ± 0.99^{b}	5.09 ± 0.31^{b}	9.29 ± 0.92^{a}	9.03** (0.001)
Hippocampus	5.25 ± 0.34^{b}	5.00 ± 0.40^{b}	6.92 ± 0.63^{a}	4.186* (0.027)
Cerebellum GM	2.75 ± 0.54	3.86 ± 0.42	3.54 ± 0.48	1.075^{ns} (0.356)
Cerebellum WM	5.00 ± 0.29^{b}	8.55 ± 0.57^{a}	9.42 ± 0.80^{a}	8.532** (0.001)
Brainstem	5.00 ± 0.90	7.96 ± 1.01	6.29 ± 0.68	2.409^{ns} (0.110)

Statistical analysis of the region wise GFAP positive cell density was carried out and the results were consolidated as shown in Table 2.

A remarkable increase in number and size of astrocytes positively marked for GFAP was observed in both forms of rabies. Statistically significant astrogliosis was noted in the white matter of the cerebellum in the case of the paralytic form ($p<0.001$). While astrogliosis was seen diffusely in the white matter of both the cerebrum and cerebellum ($p<0.001$) in the furious form of rabies, hypertrophy and significant astrocytosis in hippocampus ($p<0.027$) were also observed. There was an evident cuffing of blood vessels with the astrocytic end feet processes in both forms of rabies which was completely absent in the control group.

There was prominent hyperplasia and hypertrophy of astrocytic cells in the white matter of the brain with the presence of elongated processes intensely positive for GFAP in both forms of rabies and this finding was endorsed by the observations of the study by Headley *et al*. (2001) in a dog affected with canine distemper. Machado and Alessi, (1997) reported a higher rate of astrocytosis in brain of cattle infected with rabies. Increase in the astrocytic cytoplasm and enlargement of processes were observed in both form of rabies but at different sites; the hippocampus specifically in furious form and the cerebellum in the paralytic form. Zhan *et al.* (2017) reported the thickening and elongation of the astrocytic processes during the migration of astrocytes. Hypertrophy of the astroglial cells were specifically seen in the white matter of cerebellum in the paralytic form, and this was well evident in white matter of the brain as well as hippocampus in the case of the furious forms and this was a common observation at the areas of glial limitans in both forms of rabies. Kojima *et al.* (2009) observed a significant increase in expression of GFAP and morphological variations of astrocytes in the brain of animals infected by rabies.

The distribution of GFAP in the cerebellum of the control group of animals was seen mostly concentrated in the white matter at the tip of cerebellar folia; furious cases showed a wide distribution of GFAP processes throughout the white matter of the folia and the processes extended to the molecular layer of cerebellum as well. Headley *et al.* (2001) reported a higher amount of GFAP positive fibres in the grey matter of dogs infected with canine distemper.

The remarkable cuffing behaviour of astrocytic end feet processes was observed around the blood vessels in both forms of rabies but was however absent in control group. Astrocytes adopted a special mechanism during CNS infections such as neuronal

repair facilitation, regulation of synaptic functions and extension of astrocyte end feet processes to act as a protective barrier in guarding the blood brain barrier (Soung and Klein, 2018). Immunohistochemical studies of viral encephalitic diseases in the equine brain conducted by Delcambre *et al*. (2016) using GFAP protein revealed an abnormal increase in GFAP positive astrocytes in the brain parenchyma, near the blood vessels, at the glial limitans and around the areas of gliosis. Venugopal *et al.* (2013) reported fluctuations in the expression of proteins involved in signal transduction, energy pathways and cell growth and highlighted the invariable over expression of the astroglial marker GFAP, in encephalitis caused by rabies.

A significant increase in number of astrocytes was seen in the hippocampus of animals affected with the furious form of rabies. The dentate gyrus of the hippocampus had a remarkable increase in number and size of astrocytes in cases of the group of animals affected by the paralytic form of rabies and a similar observation in this regard was also made by Machado and Alessi (1997) who observed an increased astrocyte activity and higher GFAP immunoreactivity at the hippocampus and dentate gyrus region with large processes.

Highest immunoreactivity to GFAP was observed in 33.3 percent of cases of the furious form of rabies, while only 27.3 per cent of paralytic cases manifested the same level of intensity and this finding was comparable to that reported in the study by Headley *et al.* (2001) who suggested that acute encephalopathy expressed highest number of GFAP positive astrocytes in a given area which would become less as the disease progressed into a chronic phase.

Hol and Pekny (2015) conducted studies on the role of astrocytes and GFAP during CNS diseases and concluded that reactive gliosis was a beneficial phenomenon during a CNS insult due to the neuroprotective effect of this phenomenon ; the authors went on to suggest on possible opportunities for inventing novel treatment and prevention protocols using the same. Brat (2018) suggested that reactive astrocytosis was a response to any insult to brain, wherein these cells proliferate through an increase in the size of the cell body or processes depending on the chronicity and severity of the injury or infection.

Conclusion

Astrocytes are key cells among the glial population that provide nutritional and structural support to the neurons. Immunostaining technique has enhanced the possibility of identification of astrocytes thus permitting an evaluation of the extent of any alterations. The results of this study report on significant hypertrophy and hyperplasia of astrocytes in brain samples from dogs affected with both forms of rabies. Region wise assessment indicated that astrogliosis was seen in a more diffuse form in brains of animals afflicted with the furious form of rabies but this was localized in brain samples of animals afflicted with the paralytic form of rabies, and this was specifically into the white matter of the cerebellum. Astrocytes in the brain samples of animals afflicted with the furious form of rabies had an intense expression to GFAP when compared to those afflicted with the paralytic form. There was extensive distribution of fibrous astrocytes with elongated fibres throughout the white matter of cerebrum and cerebellum. Also significant increase in the size of protoplasmic astrocytes and thickening of the astrocyte processes in the hippocampus was evident in samples from the furious form. The regions that showed astrogliosis were different and specific in the brains of dogs infected with rabies and manifesting two different forms of rabies, though neuronal alterations were not significant. The influence of these reactive astrocytes on the pathogenesis of the disease is still a potential area for investigation.

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Virulence determinants of *Malassezia pachydermatis* isolated from cases of canine dermatitis

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Abstract:

Malassezia is a commensal organism known to cause disease under favourable conditions, and has been isolated from many animals as well as human beings. Malassezia pachydermatis is the most common cause of yeast dermatitis in dogs and there are many determinants involved in the production of disease in the host. This study aims to deterimine the presence of virulent determinants of the organisms isolated from fifteen dogs with dermatitis that may be involved in the pathogenic mechanism of Malassezia. The virulence determinants of M. pachydermatis viz; adherence, cellular surface hydrophobicity and biofilm formation were investigated. All the isolates studied were shown to produce all the virulent factors investigated in vitro which can be compared to the biological system. Adherence of organism on polystyrene plates was evident in all the isolates and the values ranged from 0.14 to 63 per cent with five isolates showing high adherence values. Hydrophobicity was variable and ranged from 1.78 to 69.46 per cent by two phase system with seven isolates showing moderate property. All the isolates were shown to produce biofilm by crystal violet staining technique and the optical density values ranged from 0.075 to 0.56 at 620 nm. No significant correlation was observed between the three virulent determinants examined. The presence of three virulent determinants investigated warrants their consideration in further studies for assessing the pathogenicity of Malassezia dermatitis in dogs.

Keywords: *Malassezia, adherence, cellular surface hydrophobicity, biofilm*

Malassezia organisms are the normal inhabitants of skin microflora in most of the warm blooded animals; the yeast is known to manifest in the host only under favourable conditions. They are known to cause certain diseases including pityriasis versicolor (PV), folliculitis, seborrheic dermatitis, dandruff, atopic dermatitis in humans and dermatitis in animals. The properties of *Malassezia* which aid in the establishment of infection in the hosts have been studied *in vitro,* to understand the mechanism and pathogenesis of the disease.

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Biofilm formation and several other determinants including adherence, cellular surface hydrophobicity (CSH) have known to cause catheter associated fungaemia in immunocompromised humans. The organism is known to cause life-threatening fungaemia and death in pre-term neonates which makes this organism one of significant public health importance. Since *M. pachydermatis* is the most commonly isolated organism from canine dermatitis, determinants such as adherence, CSH and biofilm formation are being demonstrated in the study. The understanding of virulent factors also paves the way for comprehending the resistance mechanisms exhibited by biofilm forming strains of the organism.

Materials and methods

Dogs brought to University Veterinary Hospital, Mannuthy and Kokkalai were screened for Malassezia dermatitis. Animals were examined for the presence of lesions suggestive of Malassezia infection like erythema, hyperpigmentation, greasy exudates, scaling and primary lesions of pustules, papules and macules.

Impression smears were obtained by adhesive tape method where a piece of clear one-sided cellophane adhesive tape 5.5 cm long and 2.5 cm wide was cut from a roll. The adhesive surface of the strip was placed on the skin surface and was pressed firmly once, for two or three seconds. When the tape strip was removed from the skin, the strip was placed, adhesive side down, on to a clean glass slide (Omodo-Eluk *et al*., 2003). The strip was then stained with Giemsa stain and observed under 1000X. Cytological quantification with more than two yeast cells per oil immersion objective of the microscope were selected as positive.

The samples were obtained from the skin of dogs which were positive for budding yeast cells on impression smear dermatitis by using sterile cotton swabs. The wash fluid, composed of 0.075 M phosphate buffered physiological saline, pH 7.9 containing 0.1 per cent Triton X-100 (Bond *et al*., 1995) was used for processing of the swabs. The samples were inoculated on Sabouraud dextrose agar (SDA) with chloramphenicol (HiMedia, Laboratories, Mumbai, India) for primary isolation and were incubated at 37°C for a period of 10 days (Girao *et al*., 2006). Cultural and morphological characterisation of *M. pachydermatis* was performed. Biochemical tests such as urease and catalase tests were conducted (Guillot *et al*., 1996). The inoculating medium was deprived of oil supplements to detect lipid dependency of the yeasts.

Adherence, cellular surface hydrophobicity (CSH) and biofilm formation of *M. pachydermatis* were the major virulent determinants that were studied. Adherence and CSH assay were done with some modifications (Angiolella *et al*., 2017) and biofilm formation was determined by crystal violet staining assay (Figueredo *et al*., 2012) with some modifications.

In order to examine the adherence capacity to plastic surfaces, the yeasts were grown for 72 h at 37°C in SDB, washed twice with sterile PBS (10 mM phosphate buffer, 2.7 mM potassium chloride, 137 mM sodium chloride, pH 7.4 with 0.5 per cent Tween 20) and then resuspended at 32°C in SDB. Standard inoculum of *Malassezia* was prepared at 7.5 × 10^2 cells/ml after counting the cells. After incubation for 3 h at 32°C in six-well polystyrene plates (Nest Biotech Co., Ltd) followed by extensive washing with PBS, one ml of SDA was poured into each well and allowed to solidify. After incubation for 72 h at 37°C, colonies were counted, and the results were expressed as a percentage of the inoculum size. The inoculum size for each cell suspension was confirmed by plating aliquots of the culture directly on SDA plates. {Adherence (%) = [Number of colonies in polystyrene plate/ (Number of colonies in control plate) x dilution factor] x 100}

Cellular surface hydrophobicity levels were determined by a two-phase system. The yeast cells were grown in SDB at 37°C for 72 h. Subsequently, cells were washed twice with sterile saline buffer (with 0.5% Tween 20) and resuspended in 0.05 M sodium phosphate buffer (pH 7.2) at a final concentration of 2 × 10^6 cells/ml. Cell suspension (2 ml), adjusted to an OD of 0.7 at 600nm or OD at 600 of each suspension was recorded (OD600 control).

Furthermore, the suspension was transferred to a glass tube containing 500 µl octane (Sigma Aldrich) and shaken for 1 minute using a vortex mixer. After separation of the phases, OD of the aqueous phase was measured at 600nm (OD600 after octane overlay). {Relative CSH was calculated as: [(OD at 600 control – OD at 600 after octane overlay)/OD at 600 control] × 100}

Biofilm production by single cultures of the isolates of *M.pachydermatis* was determined using a crystal violet staining method (CVS). Briefly, all isolates were grown in YEPD broth for 3 days at 32°C with intermittent shaking. After 3 days incubation, the concentration of inoculum was adjusted at 0.1 optical density at 600 nm using a spectrophotometer/ELISA reader or approximately 1.0×10^6 cell/ml. The colony forming unit (CFU) of suspension was counted on SDA. A total of 150 µl of suspension were added into 96 well flat bottom microtitre plates (Nest Biotech Co., Ltd). Thereafter, the plates were incubated for 24 h at 32°C allowing adherence phase of biofilm. Then, non-adherent cells were gently removed by double washing with 150 µl of phosphate buffered saline solution (PBS, pH7.2). After the rinsing step, a continuous culture was established by adding 200 µl of YEPD broth to each well under the previous conditions. The equal volume of YPD media were daily replaced for 4 consecutive days. To remove non-adherent yeasts, the microtiter plate wells were gently washed twice with phosphate buffered saline and fixed with 150 µl of 99 per cent methanol for 15 minutes then dried at room temperature for 45 minutes. The incubated plates were filled with a 0.5 per cent crystal violet solution for 45 minutes and washed with 200 µl of sterile distilled water, and destained with 95 per cent ethanol for 200 µl for 45 minutes. A total of 100 µl from each well was transferred to a new microtiter plate. Biofilm production was measured using the crystal violet binding assay, with the quantity of biofilm directly represented by measurement of the OD value at 620 nm in an ELISA microplate reader.

Results and discussion

The lesions were typically seen on the ventral neck and abdomen with primary and secondary skin lesions (Fig 1). Localised and generalised alopecia along with papules, erythema, crusts and excoriations were observed as the principal findings. Figure 1 shows periorbital alopecia and hyperpigmentation with lesions on the nose. Dogs with dermatitis showed clinical signs such as pruritus, hyperkeratosis, lichinification, interdigital erythema, scaly lesion, greasy seborrhoea which were similar to the signs described by Gueho *et al*. (1998), Bond *et al.* (2010) and Daniel *et al.* (2021).

Impression from cellophane tapes obtained from the skin exposed peanut shaped cells bound to the canine epithelial cells; bacterial cells were seen in pyoderma (Fig. 2). Guillot and Bond (1999) and Maynard *et al*. (2011) observed similar findings with respect to impression smears where they

Fig. 1. Malassezia hyperpigmentation

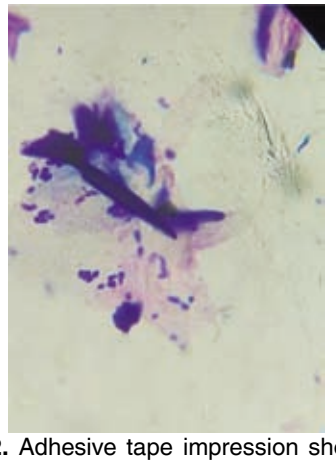

Fig. 2. Adhesive tape impression showing yeast cells on corneocytes

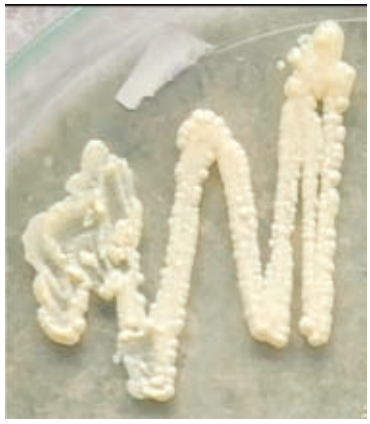

Fig. 3. Colonies appeared as ivory colored convex structures

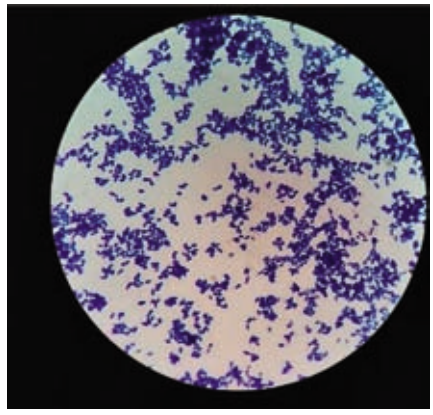

Fig. 4. Budding yeast cells X1000, Giemsa stain

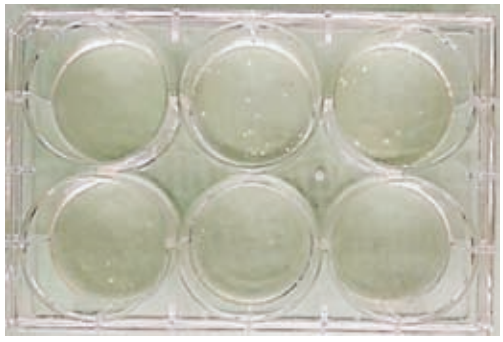

Fig. 5. Colonies on polystyrene plate

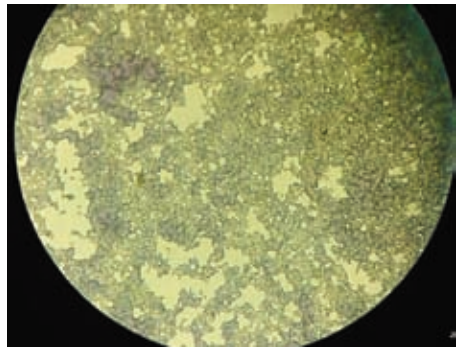

Fig. 6. Network of budding yeast cells on CVS assay X400, crystal violet stain

observed yeast cells attached to corneocyes. As the epidemiological studies and clinical signs in dogs with respect to bacterial and malassezia dermatitis intersect, it is important to differentiate both based on microbiological examinations (Shyma and Vijayakumar, 2012).

Among 45 cases of canine dermatitis with evidence of budding yeast cells on impression smear, 15 samples showed colonies typical of *Malassezia* species on SDA (without lipid supplementation).

Macroscopic appearance of colonies showed dull and rounded appearance in SDA medium after incubation for seven days (Fig.3). On microscopic examination, peanut shaped, unipolar budding yeast cells without any hyphae or pseudo hyphae were noticed (Fig. 4). The colonies were ivory coloured and convex with mean diameter of around 5 mm, positive for urease and catalase activity which was in par with Gueho *et al*. (1996). All the isolates of in the study showed growth at 37°C and 40°C as described by Ashbee and Evans (2002). Cultural, biochemical and morphological qualities were characteristic of *M. pachydermatis*.

The detergent action of Triton X-100 was found to remove the adherent yeast cells on the epidermal cell layer especially the corneocytes. Bond *et al*. (1995) observed similar findings while quantifying the yeast cells.

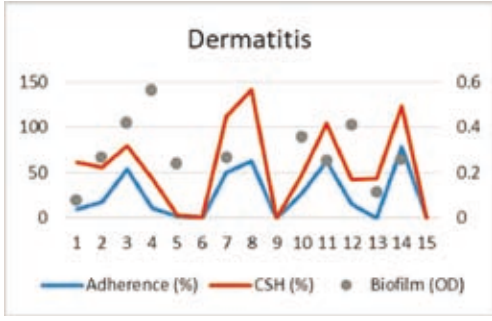

Fig. 7. Determinants in dermatitis group

All the isolates showed adherence property on polystyrene plate (Fig. 5) with five isolates showing high adherence values ranging from 50 to 78 per cent. The adherence of isolates on plastic surface was evaluated on six welled polystyrene plates and the percentage of adherence values varied from 0.14 to 78 per cent.

The property of *Malassezia* species such as adherence and cellular surface hydrophobicity helps in the formation of biofilm in living as well as inanimate objects. Attachment of *Malassezia* yeast cells on biological surface or inanimate object serves as a basis for colonisation of the organism and delay in the resolution of disease if caused. The adherence values obtained in the study are similar to the values from Angiolella *et al*. (2017) for *M. furfur* and Angiolella *et al*. (2020) for *M. sympodialis*, *M. globosa*, and *M. slooffiae*.

Results of CSH obtained by two-phase system for the isolates showed values ranging from 1.78 to 69.46 per cent. It was observed that seven isolates showed medium hydrophobicity with values 40.95 to 69.46 per cent while rest of the isolates showed low hydrophobicity values. The CSH values obtained in the study are similar to the values from Angiolella *et al*. (2017) for *M. furfur* and Angiolella *et al*. (2020) for *M. sympodialis*, *M. globosa*, and *M. slooffiae,* none of the isolates showed high CSH property. Studies have been carried out by Sivasankar *et al*. (2015) targeting the hydrophobic property of *Malassezia* thereby indirectly aiming at the treatment of *Malassezia* by decreasing the colonisation.

Biofilm formation was estimated by crystal violet staining technique, which is an indirect assessment, indicated by OD values in an ELISA microplate reader. The OD values from ELISA reader ranged from 0.075 to 0.56 at 620 nm. All the 15 isolates from dermatitis cases produced biofilm in different concentrations. In this study, all the isolates produced biofilm at various levels similar to Bumroongthai *et al*. (2016).

On ELISA plate, the organisms were seen as network of cells with clumps of budding yeasts, proving the ability of *Malassezia* to adhere and form biofilm (Fig. 6). The networking of cells was more in samples with high OD and vice-versa which was in par with Cannizzo *et al*. (2007).

Table 1. Pearson's correlation coefficient for the virulent determinants

Virulent determinants	Pearson's correlation coefficient
Adherence & CSH	0.358
CSH & Biofilm	-0.217
Biofilm & Adherence	0.036

p>0.05=not significant (ns)

The relationship between adherence, hydrophobicity and biofilm values were assessed group using the Pearson correlation. There was no statistically significant correlation between these virulent determinants tested. Pearson correlation coefficients between the virulence determinants are given in Table 1 for different groups. The values of these determinants are plotted against each other from the same sample (Fig. 7). Angiolella *et al*. (2017) had tried to correlate between CSH, adherence and biofilm formation and no statistically significant correlation was obtained which agrees with the findings of this. Regardless of this, the presence of other virulence determinants of *Malassezia* such as pigment production, lipid metabolism (Mayser and Hort, 2011), genes encoding for azelaic acid, arachidonic acid and various enzymes such as chondroitin-sulphatase, esterase, lipase, phospholipase etc. may also contribute to the pathogenicity of the disease.

Conclusion

The dogs with typical signs of Malassezia lesions were examined for the presence of virulent determinants. The *M. pachydermatis* isolates were characterised by cultural, morphological and biochemical properties. All the isolates included in the study displayed properties of adherence, hydrophobicity and biofilm formation at various intensities. Further study may be required to correlate the levels of the determinants and the disease status of the animal. No statistically significant correlation has been observed between the virulence factors tested. Biofilm helps in forming of colonies on the skin surface as well as intravenous catheters and hence

considered as the most important virulent factor among the three. Thorough study comprising the above factors and other dependent factors may be required to understand the pathogenesis of the organism.

Conflict of interest

The authors declare that they have no conflict of interest.

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Histological and ultrastructural characterisation as minimal criteria for assessing the success of the decellularisation protocols for tissue engineering applications#

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Abstract

There is a great demand of suitable biomaterials for tissue engineering applications in regenerative medicine. Decellularized extracellular matrix scaffolds derived from different organs and tissues have been successfully applied for human use as therapeutic agents in many tissue defects. The aim of the present study was to establish histological and ultrastructural characterisation as minimal criteria for assessing the suitability of decellularised bioscaffolds in tissue engineering applications. The study was performed on bovine omentum processed by a natural bovine bile based decellularisation protocol. Histological analysis and scanning electron microscopy examinations were performed and compared with an established decellularised bovine pericardial scaffold. Gross examination revealed that the decellularisation process did not alter the shape of the omentum. Histological examination confirmed the preservation of the collagenous fibres without any observable cell nuclei following decellularisation. Further, scanning electron microscopy examination demonstrated the arrangement and ultrastructure of collagen fibrils in the bioscaffolds. Our results suggest that histological and ultrastructural characterisation provide preliminary data on determining the biocompatibility of scaffolds before planning in vivo studies in tissue engineering applications.

Keywords: *Biomaterial, bovine omentum, decellularisation*

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Organ transplantation is one of the great advances in modern medicine. However, the need for organ donors is much higher than the number of people who actually donate (Panwar *et al*., 2016). Hence, research in regenerative medicine seeks alternative approaches for treatment in tissue and organ failure using extracellular matrix (ECM) (Balakrishnan-Nair *et al*., 2019). Decellularisation is a promising technique in tissue engineering where the ECM is isolated from its native cells and genetic material in order to produce a natural scaffold (Balakrishnan-Nair *et al*., 2018). An ideal decellularisation process maintains structural, biochemical and biomechanical characteristics of ECM scaffolds, which can then be recellularised to produce a functional tissue or organ. Decellularisation can be achieved using natural, chemical, enzymatic, physical or combinative methods, where each strategy has its own merits and demerits (Xing *et al*., 2015). The decellularisation process can also alter the composition of ECM which can impact the outcome of various biomedical applications (Lin *et al*., 2019).

A properly decellularised ECM based bioscaffold preserves the amount of collagen, pore size, glycosaminoglycans (GAGs) and growth factors (Allen *et al*., 2010). Thus, these scaffolds improve the success rates in various clinical applications. However, poorly decellularised ECM bioscaffolds produce adverse immune reactions leading to rejection of the materials (Aamodt and Grainger, 2016). There is a need for establishing minimal criteria for assessing the success of decellularisation protocols. Hence, the present study proposes minimal criteria for assessing the success of the decellularised bioscaffolds based on histological, histochemical and ultrastructural studies for tissue engineering applications in repairing bioscaffolds. The ultimate aim of tissue engineering is to develop low cost and effective next generation "off the shelf" ECM based solution for clinical applications.

Materials and methods

Preparation of scaffolds

Fresh bovine omenta were collected from Meat Technology Unit, College of Veterinary and Animal Sciences, Mannuthy at the time of slaughter. Manual delamination was done to separate omental layers. Defattening was achieved by manual and chemical (chloroform: methanol; 2:1) methods. Defattened omental scaffolds were subjected to decellularisation process with 80 per cent aqueous bile in a shaker incubator followed by washing with 1 per cent Tris Buffered Saline with Tween-20 (TBST) and later dried under laminar air flow (Ashna *et al*., 2019). The sheets were well packaged in a polythene bag and sterilised by gamma irradiation at 25KGy (BRIT, Mumbai, India) (Ashna *et al*., 2019). Decellularised bovine pericardium procured from Division of *In Vivo* Models and Testing, Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram, was used as control.

Microscopic evaluation

Representative samples of decellularised bovine omentum and decellularised bovine pericardium were fixed in 10 per cent neutral buffered formalin solution (10% NBF). These scaffolds were then washed and dehydrated, embedded in paraffin wax and sectioned at 4-5µm thickness. The sections were stained with haematoxylin and eosin (H&E), Masson's trichrome (connective tissue stain) and alcian blue stains (glycosaminoglycans) (Suvarna *et al*., 2018).

Scanning electron microscopy (SEM)

The representative samples of decellularised bovine omentum and decellularised bovine pericardium were fixed in 2.5 per cent glutaraldehyde solution for 48 h. The samples were then washed thrice with buffer (0.1 M PBS) for 10 min each. The samples were then dehydrated through ascending grades of alcohol (50%, 60%, 70%, 80%, 90% and 100 %) for 45 min each. The samples were air dried and mounted on sample stub having double sided carbon adhesive. Later, the samples were sputter coated with a thin layer of gold and analysed using scanning electron microscope (Hitachi 3000N) at an accelerated voltage of 15kV and micrographs were taken.

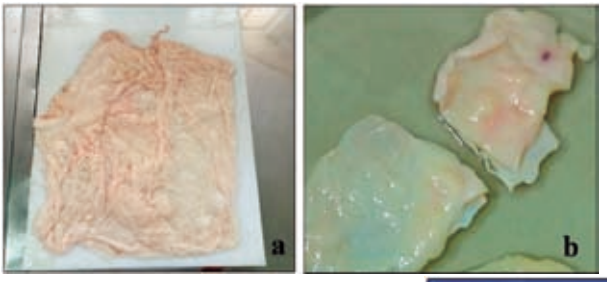


Fig. 1.
a. Fresh bovine omentum
b. Fresh bovine pericardium

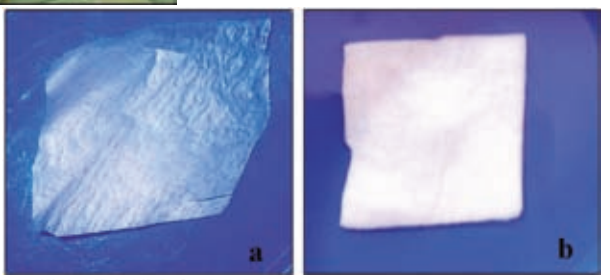


Fig. 2.
a. Decellularised bovine omentum
b. Decellularised bovine pericardium

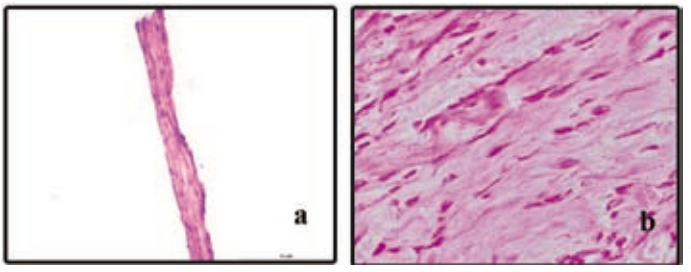
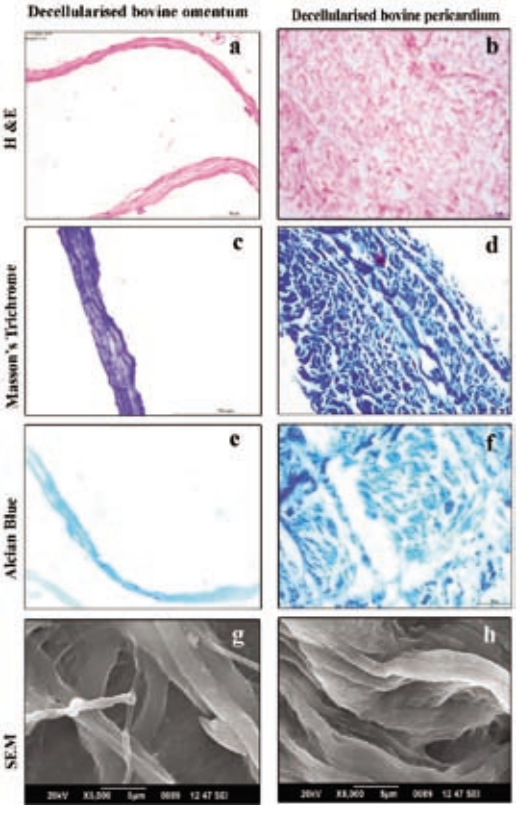


Fig. 3. a. Non decellularised bovine omentum showing nucleus in the matrix (H&E X 400) b. Non decellularised bovine pericardium showing nucleus in the matrix (H&E X 400)

Fig. 4.

a. Decellularised bovine omental scaffold showed pink coloured cytoplasm with absence of nuclear remnants (H&E X 400) b. Decellularised bovine pericardial scaffold showed pink coloured cytoplasm with absence of nuclear remnants c. Decellularised bovine omental scaffold showed parallel collagen pattern with absence of nuclear remnants (Masson's Trichrome X 400), d. Decellularised bovine pericardiual scaffold showed parallel collagen pattern with absence of nuclear remnants (Masson's Trichrome X 400),e. Decellularised bovine omental scaffold showed presence of glycosaminoglycans in the decellularised matrix (Alcian blue stain X 400), f. Decellularised bovine pericardial scaffold showing presence of glycosaminoglycans in the decellularised matrix (Alcian blue stain X 400), g. Scanning electron micrograph of decellularised bovine omentum revealed acellular, intact and thick collagen fibers and pores were visible (red arrow)(SEM X 5000) h. Scanning electron micrograph of decellularised bovine pericardium revealed acellular collagen fibers and pores (red arrow) (SEM X 5000)



Results and discussion

Decellularised omenta were prepared from native tissues (Fig. 1) using a natural bile based decellularisation protocol (Fig. 2). Hematoxylin and eosin (H& E) staining was performed on decellularised bovine omentum and decellularised bovine pericardium (Fig. 3). No nuclei or cell fragments were visible in both decellularised scaffolds up on H&E staining. In Masson's trichrome staining, blue coloured collagen was observed in decellularised bovine omental and pericardial scaffolds with the absence of black coloured nuclei (Fig. 2). Presence of glycosaminoglycans (GAGs) was demonstrated with alcian blue staining in decellularised bovine omental and pericardial scaffolds. In both decellularised bovine omental and pericardial scaffolds, the presence of GAGs was indicated in light blue color (Fig. 4). Scanning electron microscopy demonstrated collagen pattern and pores in between the collagen fibers of various scaffolds (Fig. 4). The decellularised bovine omental scaffolds and decellularised bovine pericardial scaffolds showed acellular wavy collagen pattern with sufficient pores in between collagen fibres.

Decellularisation is a critical point in tissue engineering which affect the architecture and composition of biomaterials. Different decellularisation protocols have been established to yield several tissue derived scaffolds for tissue engineering applications. According to Vasudevan *et al*. (2016), the bile decellularisation method was proven to be successful for removing most of the cellular contents from the matrix of omentum. Bovine bile is an abattoir waste that can be used as good decellularising agent for ECM matrices because of its detergent property attributed to the contents such as sodium cholate and sodium deoxycholate (Azum *et al*., 2019). This is *via* altering lipid membrane structure of cells leading to dispersion of lipid contents of cell membrane resulting in destruction of cells and effective removal of cells from the ECM matrix leaving scaffold network. After decellularisation, an ideal ECM scaffold is also expected to maintain the structure of collagen and elastin fibers as well as content of biochemical molecules such as GAGs. It has been noted that any disturbance caused in the architecture of collagen fibres in ECM can affect biomechanical properties of the scaffolds (Sheridan *et al*., 2012).

Functional and structural disturbances of ECM were not only associated with collagen fibres but also related to elastin degradation, glycosaminoglycans loss etc (Chen *et al*., 2004). Physical (freeze-thawing cycles, mechanical forces), chemical (ionic and non-ionic detergents; hypertonic or hypotonic salt solutions, and acids and bases) and biological methods (enzymes) have been extensively used for decellularisation (Dussoyer *et al*., 2020). In addition, nucleases are used to promote the fragmentation of residual DNA into <200 bp fragments in order to minimise immunological responses (Fernandez-Perez and Ahearne, 2019). Decellularisation was said to be effective when DNA fragment length was between 100- 200 base pair and also DNA dry weight in the decellularised matrix was less than 50ng. Extensive research has been conducted to optimize these decellularisation procedures to allow for maximal cell removal and minimal ECM damage for each tissue/ organ. The present study was to establish histological and ultrastructural characterisation as minimal criteria for assessing the success of the decellularised bioscaffolds following various Decellularisation protocols for tissue engineering applications. Decellularisation can remove cellular components; otherwise, which can induce residual immunological response in host (Wong and Griffiths, 2014). Bile is an emerging decellularising agent owing to its chemical composition having decellularisation property. The efficiency of decellularisation process was studied by considering the absence of cell nuclei with H&E (He, 2020). Here, both scaffolds upon H&E staining showed absence of cellularity confirmed the effectiveness of decellularisation (Fig.2). Masson's trichrome staining revealed preservation of the collagenous fibres (Fig. 2), as well as porosity. Alcian blue staining was used to ascertain the presence GAGs in decellularised matrix (Fig. 2). Retention of naturally occurring GAGs in the decellularised matrix can act as a factor for cell growth due to its ability to bind growth factors and cytokines

and also owing to its water retention property (Xing *et al*., 2014). Glycosaminoglycans have important role in growth, migration and proliferation of cells (Salbach *et al*., 2012).

In the present study, scanning electron microscopy images revealed microarchitecture of acellular collagen fibres with sufficient pore sizes in both decellularised bovine omental and pericardial scaffolds which is beneficial for recellularisation and cell growth (Fig. 2). Both decellularised bovine omental and pericardial scaffolds showed acellular, intact and thick collagen fibres, with sufficient pores size. According to the observations made by Porzionato *et al*. (2013) decellularisation of omentum resulted in removal of lipid and cellular components from the matrix but preserved the architecture of collagen, elastin and reticular fibres and glycosaminoglycans needed for regeneration applications. We therefore suggest histological and ultrastructural characterisation of ECM bioscaffolds as the most basic and minimal criteria for assessing the efficiency of decellularisation protocols. Advanced immunological assessment of scaffolds by means of special nuclear stains like DAPI, Hoechst 33342, Hoechst 33258 etc and DNA quantification employing nanodrop spectrophotometry can be further employed for confirmation.

Conclusion

In future studies, we can develop cell quantification approach as a strategy using H&E stained sections for tissue engineering applications. In the present study, histological and ultrastructural characterisation provide preliminary data on determining the biocompatibility of scaffolds before planning biomechanical testing or *in vivo* studies for tissue engineering applications. Hence, histological and ultrastructural characterisation can be considered as minimal criteria for assessing the success of the decellularisation protocols for tissue engineering applications.

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Conflicts of interest

The authors declare that there is no conflicts of interest**.**

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Organoleptic evaluation of Ladakhi *churpe* enriched with apricot and spinach

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Abstract

Dried dairy products viz. churpe-balls and churpe-strips were developed with the incorporation of apricot powder into cottage cheese at different levels (05, 10, 15 and 20%) and spinach powder at levels 03, 06, 09 and 12 percent, respectively. The sensory attributes of the products were studied during the investigation. The appearance of balls first decreased, then increased and again decreased and that of strips first increased and then decreased. The supplementation resulted in significant (P < 0.05) decline in texture of both products. Flavor increased in balls and decreased in strips with the addition of supplements. Overall acceptability first got increased and then decreased with the increase in the level of supplementation of the products. The titratable acidity increased whereas pH decreased in both products upon the incorporation of additives. Storage studies done for 360 days reveal that except texture and titratable acidity, there were losses in all the parameters in both products with the advancement of storage. Except flavor in case of balls and overall acceptability and pH in case of strips, all the interaction effects of supplementation and storage were found to be statistically significant.

Keywords: *Churpe, Ladakh, apricot, spinach, churpe-balls, churpe-strips*

Churpe is a traditional dried dairy product which is very much popular in Himalayan regions of Nepal, Bhutan, Tibet and India. In India, it is consumed in Sikkim, Assam, Darjeeling, Kalimpong and Ladakh. It is a nutrient capsule for the tribal people of these regions and also has tremendous health benefits. In Ladakh the fresh cheese is shaped into small strips and dried in open sun. However in other regions it is moulded into cuboidal pieces and hung over fire to harden it.

In Ladakh, *churpe* is generally prepared during summer when the milk production is surplus, for consumption during harsh winter season when the region got cut off from outside world due to heavy snowfall. The purpose of drying cheese is mainly to increase the storability

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which is not possible in the fresh products. Local people consume it either by cooking with *thukpa*, a thick soup or by chewing it in mouth and masticate to get its health benefits as well as to enjoy its characteristic flavour. Its flour is mixed with *kholaq* and *chasrul* and is also an important ingredient of *thut*, a sweet. *Kholaq* is a traditional dish in which the roasted barley flour, *namphey*, is mixed with tea to a consistency, where it does not stick to the hand. It does not require cooking. When *namphey* is added to tea up to a consistency thicker than soup, then it is called as *chasrul*. Therefore, *churpe* is not only a food product but also an integral part of food culture of Ladakhis. Like other dairy products, *churpe* is also considered as a nutrient capsule containing quality proteins, vitamins and minerals. The probiotic properties of indigenous microorganisms isolated from the cheese-like product *churpe* have been reported (Tamang *et al*., 2000). Probiotic milk products can have health-promoting benefits such as modulation of the immune system, maintenance of gut flora, regulation of bowel habits, alleviation of constipation, and curing of gastrointestinal infections (Tamang, 2010). Yeast, mold, lactic acid bacteria (LAB), and Bifidobacterium species do not play an important role in *churpe* preparation but their synergistic actions convert the milk sugar into beneficial compounds, such as vitamins, lactic acid, etc. (Panda *et al*., 2016). However, it lacks polyphenols like fruits and vegetables which play important role as antioxidants in the human body. It has been reported that fortification of food products using natural resources like fruits, vegetables, herbal extracts, cereals, nuts, seeds, etc. is necessary to improve nutrient intake (Granato *et al*., 2017).

Apricot is the major fruit crop of Ladakh which is the only source of income to a large section of population (Hussain *et al*., 2012; Hussain *et al*., 2013). Spinach is also one of the major vegetable crops of Ladakh. These crops in their dried form provide nutrients to the nutrient-deficient people during the winter months when the region remains cut off from the outside world due the closure of roads because of heavy snowfall. Therefore, the aim of this study was to produce novel *churpe* products with apricot and spinach supplementation. In the current investigation, the effect of additives on the sensory attributes of *churpe* during storage was investigated. Cheese snacks as cheese balls and chips are very popular worldwide (Rakcejeva *et al*., 2009). The value-added products developed were apricot added *churpe*-balls and spinach added *churpe*-strips. These products can be used to solve the problem of nutrient deficiency (Wiley 2004; Attenborough *et al*., 1994; Cvejic *et al*., 1997) among this tribal population of Ladakh.

Materials and methods

Materials

The raw buttermilk produced from cow milk was procured from the herders of Nyoma and Nidder villages of Changthang region, Leh Ladakh. Dried apricots without stone were procured from the local market of Leh. Fresh spinach leaves (moisture content 94.8%) were obtained from Vegetable Farm, Krishi Vigyan Kendra, Nyoma (Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir).

Drying of spinach

The destalked spinach leaves were washed with tap water. Before drying, the leaves were blanched for 15 seconds at 98°C with a spinach-water ratio of 1:4 with slight modification of blanching time as recommended by Sharma *et al*. (2011). The blanched leaves were then shade dried.

Preparation of products

The method given by Hussain *et al*. (2022) was followed for the preparation of the value added dairy products. Dried spinach and dried apricot were converted into powder in a food processor (HR-7629, Philips, China). A sieve with 750 µm pore size was used to sieve the powders. The buttermilk was boiled for 10 min at 70 °C and the coagulum so obtained was subjected to cooling. The holding time in whey was about 5 minutes. The solid mass (cottage cheese) was separated from the whey by straining through a cheese cloth. It is then incorporated with the additives, apricot powder for balls and spinach powder for strips. Since

balls were to be eaten as such thus apricot being a fruit was added and strips were first to be cooked in *thukpa* thus spinach was added. Blends were prepared by replacing the mass with apricot powder at 5%, 10%, 15% and 20% with 10% ground sugar for *churpe*-balls. For *churpe*-strips, the mass was replaced with spinach powder at 3%, 6%, 9% and 12%. The mixture was then kneaded. Balls were made by rolling the mass between the palms. Strips were made by pressing the mass between the palm and fingers. The products so obtained were dried in a solar *churpe* dryer for 3 days. After drying, the products were stored in cotton bags at an average temperature 25 ± 2 ºC and relative humidity of 30 %. The *churpe* strips without any addition were taken as a control in this study.

Proximate composition

Analysis of moisture, protein, fat, and ash contents of the samples were performed according to the method described by the Association of Official Analytical Chemists (AOAC, 1990).

Lactose

Titrimetric method as described by Adolf Lutz Institute (2005) using Fehling licor (solution containing cupric ions in alkaline medium) was applied to measure lactose in the products. A solution of each product (balls and strips) was made using 50 g dissolved in 2 mL acetic acid (2% v/v) and distilled water. The mixture was heated for 5 minutes at 80°C. After this, the samples were transferred to volumetric flask of 200 mL and volume was completed with distilled water. After filtration, the solutions obtained were used to react with 20 mL of standard Fehling licor.

Minerals

The determination of calcium, sodium and magnesium was carried out by flame photometry (direct method) as given by Kravic*et al*. (2012) using flame photometer (Systronics, India) in air-butane flame. After the homogenization by mixing, 2.5 g of the sample was transferred to a calibrated flask, 2 cm^3 of 10% solution of lanthanum was added and diluted to a final volume of 50 cm^3 with distilled water, filtered and the resulting solution was used for the analysis.

Hydrosoluble vitamins

The protocol given by Ghosh *et al*. (2015) was followed for the quantification of hydrosoluble vitamins. These vitamins were analyzed by reverse phase-HPLC using an Agilent HPLC system (Agilent Technology) equipped with a Zorbax SB-C18 column and the mobile phase was 0.05MKH_2PO_4 (pH 2.5) and acetonitrile (A). The solvent gradient was as follows: at 0 minutes 0.6% A, at 0.5 minutes 0.6% A, at 4 minutes 0.6% A, at 12 minutes 0.6% A, at 17 minutes 0.6% A, and the stop timewas 20 minutes. The temperature was kept at 15 °C and a constantflow rate of 1 mL/min was maintained. The effluent from the column was monitored by variable wavelength UV detector (204 nm).

Antioxidant activity

The antioxidant activity was determined by DPPH (1,1, diphenyl-2-picryihydrazyl) scavenging activity using DPPH as a free radical as per the method given by Brand-Williams *et al*. (1995). 100 µl of sample extract solution was added to 1ml of 0.01 percent methanolic solution in a cuvette. The sample was then incubated for 30 minutes at room temperature. The reaction solution was examined at 515 nm using a spectrophotometer. The inhibition percentage of DPPH solution was calculated according to the below equation:

$$\text{Inhibition (\%)} = \frac{(Abs_{t0min} - Abs_{t30min})}{Abs_{t0\ min}} \times 100$$

Where, $Abs_{t0\ min}$ = absorbance of DPPH at initial stage and $Abs_{t30\ min}$ = absorbance of DPPH after 30 minutes of incubation

Titratable acidity

Acidity in dried cheese products was estimated by Titration Method No.920.124 of AOAC (1990). 1 g of each cheese sample was mixed with warm water and volume was made up to 10 mL in 100 mL conical flask; Sample containing flask was shaken vigorously and filtered. The

filtrate was titrated with 0.1 N NaOH using phenolphthalein as indicator. The percent of titratable acidity was calculated according to the following expression:

$$\text{Titratable acidity (\%)} = \frac{0.0090 \times \text{volume of NaOH used} \times 100}{\text{Weight of sample}}$$

pH

The pH of the products was estimated according to the method of Panda *et al*. (2016). The pH of the product (10 g) was determined by homogenizing the sample with sterile distilled water (100 mL) in a ratio of 1:10, followed by shaking for 5 minutes. The pH of the fermented substrate was then measured by a glass probe digital pH meter (Eutech, Singapore).

Storage studies

The developed products were packed in cotton bags and stored for a period of 360 days at ambient temperature (25 ± 2 ºC). The stored products were analyzed for various sensory properties at an interval of 90 days following the standard procedures.

Sensory evaluation

The organoleptic attributes of the value-added dairy samples were assessed by semi-trained panelists comprising of professionals, non-professionals and consumers using the evaluation criteria described by Demirci *et al*. (2017). The panel was asked to evaluate the samples using a 9-point Hedonic scale (1, Very bad; 2, Bad; 3, Imperfect; 4, Sufficient; 5, Mediocre; 6, Satisfactory; 7, Good; 8, Very good; and 9, Excellent) for appearance, flavor, texture, and overall acceptability. The balls were analyzed as such but the strips were soaked in water prior to analysis because the strips are usually consumed after cooking.

Statistical analysis

Results of determinations reported in this study constitute a mean from three replications. For the purpose of objectivity of inference, the recorded results were subjected to statistical analysis. For the determination of significance of differences between means, analysis of variance (ANOVA) was conducted using the OP-Stat software (Version 1.0). Dependencies were considered statistically significant at the level of significance $P < 0.05$.

Results and discussion

Nutritional composition

On the basis of organoleptic study, 15% and 9% incorporations of apricot and spinach, respectively, gave best results and were selected for analysis of nutritional composition of value added *churpe* products. The nutritional composition of the *churpe* (control), *churpe*-balls and *churpe*-strips have been shown in Table 1.

The effect storage period of 360 days on *churpe* (control) (Fig.1), *churpe*-balls (Fig. 2) and *churpe*-strips (Fig.3) are discussed below.

Sensory properties

Appearance of the *churpe* added with apricot and spinach powders are given in Table 2. Supplementation shows significant ($P < 0.05$) effect on the appearance of the value-added dairy products. The mean appearance score of the *churpe*-balls first decreased from 7.20 (at 5%) to 7.10 (at 10%), then increased upto 7.60 (at 15%) and then again decreased upto 5.10 (at 20%) with the increase in the level of supplementation of apricot. Appearance also increased in papaya and watermelon added yogurt (Roy *et al*. 2015). The mean appearance score of *churpe*-strips increased with increase in the level of supplement (spinach) from 7.10 (at 3%) to 8.20 (at 6%) followed by decrease upto 6 (at 12%). Sharma *et al*. (2011) observed significant decrease in appearance scores of broccoli-cheese powder blends. Storage studies revealed that the mean appearance scores of balls and strips decreased from 7.70 to 6.10 and 8.0 to 6.40, respectively, after 360 days (12 months). Appearance decreased during storage of fruit-based yogurt (Roy *et al.* 2015) and Whey Protein Concentrate-70 (Rathour *et al*., 2017).

A decreasing trend in texture was shown by both products with the increase in

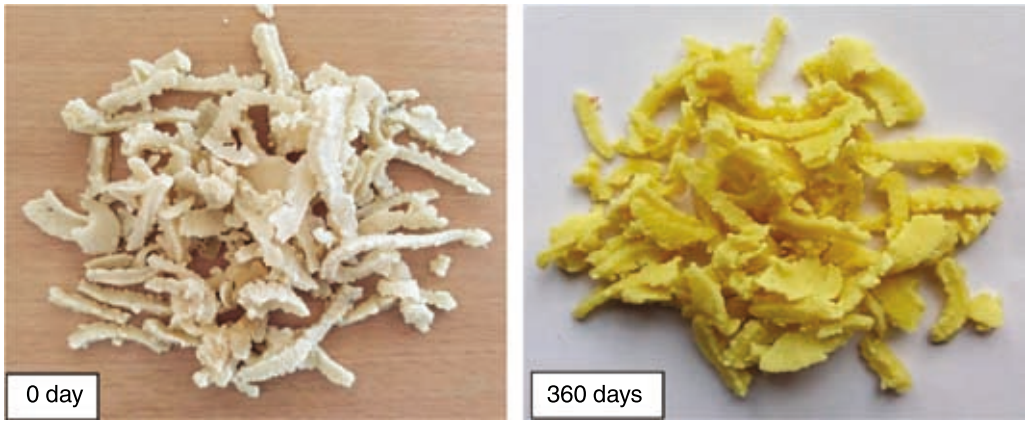

Fig. 1. Effect of storage period on *Churpe* (Control)

Fig. 2. Effect of storage period on *Churpe*-balls

Fig. 3. Effect of storage period on *Churpe*-strips

the levels of the additives as depicted in Table 3. The mean texture score decreased from 6.1 to 5.1 at 5% and 20% apricot incorporations respectively, in case of *churpe*-balls. This might be due to increase in the hardness of the products because of apricot. Mean texture score also decreased from 7.5 to 6.6 at 3% and 12% spinach incorporations, respectively, in case of *churpe*-strips. Similar observations were found by other workers for the changes in texture due to the incorporation of spinach powder (Lucera *et al*., 2018). Loose texture (low firmness) could be due to higher concentration of spinach powder which has fibrous attribute. With the advancement of storage period (360 days), the mean texture scores of balls increased from 5.4 to 6.2 and of strips increased from 7.2 to 7.6.

Table 1. Nutritional composition of *churpe* and value added *churpe* products

Parameters	*Churpe*	*Churpe*-balls	*Churpe*-strips
Moisture (%)	07.43 ± 0.10	07.48 ± 0.26	07.50 ± 0.15
Lactose (%)	42.29 ± 0.29	38.04 ± 0.14	40.82 ± 0.14
Protein (%)	36.16 ± 0.14	29.56 ± 0.06	35.71 ± 0.18
Fat (%)	07.65 ± 0.16	05.12 ± 0.21	06.89 ± 0.18
Ash (%)	07.02 ± 0.21	08.12 ± 0.17	08.69 ± 0.18
Titratable acidity (%)	06.27 ± 0.11	06.67 ± 0.26	06.68 ± 0.19
pH	04.17 ± 0.23	04.12 ± 0.07	04.68 ± 0.18
Thiamine (µg/g)	37.02 ± 0.21	37.57 ± 0.28	37.69 ± 0.18
Riboflavin (µg/g)	106.16 ± 0.14	106.56 ± 0.06	106.71 ± 0.18
Ascorbic acid (µg/g)	10.43 ± 0.10	10.48 ± 0.26	11.50 ± 0.15
Antioxidant activity (%)	37.16 ± 0.13	47.72 ± 0.21	41.28 ± 0.16
Calcium (mg/100g)	616.14 ± 0.21	608.43 ± 0.15	780.52 ± 0.09
Magnesium (mg/100g)	530.66 ± 0.16	460.63 ± 0.10	601.74 ± 0.17
Sodium (mg/100g)	883.47 ± 0.18	689.13 ± 0.03	832.57 ± 0.08

The experiments were carried out thrice and the values are represented as mean ± standard deviation, n = 3.

Table 2. Effect of supplementation and storage on appearance of value-added *churpe* products (balls and strips)

Supplementation level (%)	Storage period (days)					
	0	30	60	90	120	Mean
Control (Cottage cheese)	08.30 ± 0.18	08.00 ± 0.18	07.40 ± 0.28	07.00 ± 0.13	06.50 ± 0.28	07.40 ± 0.21
CC:AP:GS						
90:00:10	08.30 ± 0.15	08.00 ± 0.28	07.40 ± 0.21	07.00 ± 0.27	06.50 ± 0.38	07.40 ± 0.25
85:05:10	07.50 ± 0.15	07.50 ± 0.26	07.40 ± 0.20	07.20 ± 0.37	06.70 ± 0.17	07.20 ± 0.23
80:10:10	08.00 ± 0.27	07.70 ± 0.13	07.30 ± 0.12	06.60 ± 0.32	06.20 ± 0.17	07.10 ± 0.20
75:15:10	08.50 ± 0.22	08.00 ± 0.10	07.70 ± 0.19	07.00 ± 0.30	06.80 ± 0.21	07.60 ± 0.20
70:20:10	06.30 ± 0.15	05.50 ± 0.25	04.90 ± 0.20	04.70 ± 0.25	04.30 ± 0.26	05.10 ± 0.22
Mean	07.70 ± 0.18	07.30 ± 0.20	06.90 ± 0.18	06.50 ± 0.30	06.10 ± 0.23	06.90 ± 0.22
CD (P < 0.05)	Supplementation = 0.15		Storage = 0.16		Supplementation x Storage = 0.37	
CC:SP						
97:03	08.00 ± 0.20	07.70 ± 0.16	07.30 ± 0.14	06.60 ± 0.29	06.20 ± 0.20	07.10 ± 0.19
94:06	08.50 ± 0.31	08.50 ± 0.29	08.40 ± 0.26	08.20 ± 0.17	07.70 ± 0.21	08.20 ± 0.24
91:09	08.50 ± 0.12	08.00 ± 0.31	07.70 ± 0.24	07.00 ± 0.19	06.80 ± 0.28	07.60 ± 0.22
88:12	07.00 ± 0.32	07.00 ± 0.37	06.00 ± 0.17	05.20 ± 0.34	05.00 ± 0.14	06.00 ± 0.26
Mean	08.00 ± 0.23	07.80 ± 0.28	07.30 ± 0.20	06.70 ± 0.24	06.40 ± 0.20	07.20 ± 0.23
CD (P < 0.05)	Supplementation = 0.17		Storage = 0.17		Supplementation x Storage = 0.39	
CD (P < 0.05) of all treatments						

CC = Cottage cheese, AP = Apricot Powder, GS = Ground sugar, SP = Spinach powders, n = 3.

The data presented in Table 4, reveals that the flavour got enhanced in *churpe*-balls and declined in *churpe*-strips with the addition of apricot powder and spinach powder, respectively. Sucrose, γ-decalactone, β-Ionone and citrate are the key flavoring compounds while alcohols are identified as the main volatiles compounds present in apricot that contribute to the consumer acceptance of the balls (Xi *et al*., 2016). The presence of fibre in spinach is responsible for savory-sour taste of the *churpe*-strips. The mean flavor score ranged between 6.9 and 8.2 in *churpe*-balls and the same ranged between 6.4 and 4.8 in *churpe*-strips. Flavour increased in fruit added yogurt with the increase in the levels

Table 3. Effect of supplementation and storage on texture of value-added *churpe* products (balls and strips)

Supplementation level (%)	Storage period (days)					
	0	90	180	270	360	Mean
Control (Cottage cheese)	06.70 ± 0.27	06.80 ± 0.20	06.80 ± 0.18	07.10 ± 0.20	07.30 ± 0.21	06.90 ± 0.21
CC:AP:GS						
90:00:10	06.20 ± 0.32	06.50 ± 0.12	06.80 ± 0.11	07.40 ± 0.21	07.60 ± 0.11	06.90 ± 0.17
85:05:10	05.90 ± 0.21	05.90 ± 0.14	06.00 ± 0.28	06.30 ± 0.24	06.50 ± 0.17	06.10 ± 0.20
80:10:10	05.30 ± 0.35	05.30 ± 0.17	05.60 ± 0.31	05.90 ± 0.28	06.00 ± 0.15	05.60 ± 0.25
75:15:10	05.00 ± 0.18	05.20 ± 0.18	05.30 ± 0.28	05.70 ± 0.18	05.70 ± 0.19	05.30 ± 0.20
70:20:10	05.00 ± 0.25	05.00 ± 0.14	05.00 ± 0.16	05.20 ± 0.15	05.50 ± 0.10	05.10 ± 0.16
Mean	05.40 ± 0.26	05.50 ± 0.15	05.70 ± 0.22	06.10 ± 0.21	06.20 ± 0.14	05.80 ± 0.19
CD (P < 0.05)	Supplementation = 0.15		Storage = NS		Supplementation x Storage = 0.34	
CC:SP						
97:03	07.70 ± 0.14	07.80 ± 0.15	07.80 ± 0.29	07.00 ± 0.13	07.30 ± 0.13	07.50 ± 0.16
94:06	07.20 ± 0.18	07.40 ± 0.23	07.50 ± 0.38	07.60 ± 0.32	07.70 ± 0.10	07.40 ± 0.24
91:09	06.90 ± 0.14	06.90 ± 0.16	07.00 ± 0.17	07.30 ± 0.19	07.50 ± 0.20	07.10 ± 0.19
88:12	06.30 ± 0.23	06.30 ± 0.27	06.60 ± 0.28	06.90 ± 0.39	07.00 ± 0.30	06.60 ± 0.27
Mean	07.20 ± 0.17	07.30 ± 0.20	07.40 ± 0.20	07.40 ± 0.25	07.60 ± 0.18	07.40 ± 0.22
CD (P < 0.05)	Supplementation = 0.16		Storage = 0.16		Supplementation x Storage = 0.38	

CC = Cottage cheese, AP = Apricot Powder, GS = Ground sugar, SP = Spinach powder, n = 3

Table 4. Effect of supplementation and storage on flavour of value-added *churpe* products (balls and strips)

Supplementation level (%)	Storage period (days)					
	0	90	180	270	360	Mean
Control (Cottage cheese)	05.60 ± 0.18	05.20 ± 0.18	05.00 ± 0.28	04.70 ± 0.28	04.50 ± 0.12	05.00 ± 0.20
CC:AP:GS						
90:00:10	07.20 ± 0.28	07.00 ± 0.15	06.80 ± 0.38	06.20 ± 0.21	06.00 ± 0.18	06.60 ± 0.24
85:05:10	07.50 ± 0.13	07.30 ± 0.27	07.20 ± 0.17	06.40 ± 0.12	06.20 ± 0.24	06.90 ± 0.18
80:10:10	08.00 ± 0.10	08.00 ± 0.22	07.80 ± 0.21	07.00 ± 0.19	06.90 ± 0.17	07.50 ± 0.17
75:15:10	08.50 ± 0.26	08.30 ± 0.15	08.30 ± 0.17	07.40 ± 0.20	07.10 ± 0.18	07.90 ± 0.19
70:20:10	08.70 ± 0.25	08.50 ± 0.15	08.30 ± 0.26	07.90 ± 0.20	07.80 ± 0.28	08.20 ± 0.22
Mean	07.90 ± 0.20	07.80 ± 0.18	07.60 ± 0.23	06.90 ± 0.18	06.80 ± 0.21	07.40 ± 0.20
CD (P < 0.05)	Supplementation = 0.14		Storage = 0.15		Supplementation x Storage = NS	
CC:SP						
97:03	07.00 ± 0.16	06.70 ± 0.20	06.40 ± 0.20	06.00 ± 0.14	05.90 ± 0.25	06.40 ± 0.19
94:06	07.20 ± 0.29	06.50 ± 0.31	05.90 ± 0.21	05.70 ± 0.26	05.00 ± 0.24	06.00 ± 0.26
91:09	06.00 ± 0.31	05.60 ± 0.12	05.20 ± 0.28	04.70 ± 0.24	04.30 ± 0.29	05.10 ± 0.24
88:12	05.60 ± 0.37	05.00 ± 0.32	04.80 ± 0.14	04.60 ± 0.17	04.30 ± 0.27	04.80 ± 0.25
Mea n	06.70 ± 0.28	06.20 ± 0.23	05.80 ± 0.20	05.50 ± 0.20	05.10 ± 0.26	05.80 ± 0.23
CD (P < 0.05)	Supplementation = 0.17		Storage = 0.17		Supplementation x Storage = 0.39	

CC = Cottage cheese, AP = Apricot Powder, GS = Ground sugar, SP = Spinach powder, n = 3

of additives (Roy *et al*. 2015).Sharma *et al*. (2011) observed significant decrease in flavour score of broccoli-cheese powder blends. The mean flavour scores declined from 7.9 to 6.8 in case of balls and from 6.7 to 5.1 in case of strips during the storage period of 360 days. Do Boer *et al*. (1977) and Rathour *et al*. (2017) studied the deterioration of flavour of whey protein concentrate during storage. Flavour of spinach added ultra-filtrated-soft cheese

decreased during storage (El-Sayed, 2020). Flavour decreased during storage of fruit-based yogurt (Roy *et al.* 2015). The off flavor in stored product may be due to lipid oxidation and Maillard browning (Ferretti and Flanagan, 1972; Min *et al*. 1990).

The overall acceptability of the *churpe*-balls and *churpe*-strips shows an increasing trend with increase in the level of incorporation (Table 5). The mean overall acceptability score of balls increased from 6.7 to 8.1 and then decreased to 5.8 corresponding to 5%, 15% and 20% levels of incorporations, respectively, while, it got increased from 5.3 to 6.9 and then decreased to 6.3 corresponding to 3%, 9% and 12% levels, respectively, in case of strips. A similar trend was also found by Mohamed and Shalaby, (2016) in apricot-cheese analogue. Feng *et al*. (2019) showed a potential improvement in sensory properties of goat yogurt supplemented with jujube pulp. Overall scores were highly acceptable in low concentration of spinach supplemented soft cheese (El-Sayed, 2020) and biscuits (Narsing *et al*., 2017). The mean overall acceptability scores decreased from 7.5 to 6.1 in balls and from 6.9 to 5.5 in strips after 12 month (360 days). Overall acceptability decreased during storage of fruit-based yogurt (Roy *et al*. 2015). Decrease in sensory scores with the increase in storage period could be explained as a result of interactions of all the physico-chemical parameters. The changes in multiplicity of physico-chemical parameters like contents of moisture, hydroxymethyl furfural (HMF), free fatty acid (FFA) and reflectance governed the sensory scores of the product. Storage temperature and time, relative humidity and gaseous concentration in storage environment are major factors responsible for degradation of the sensory characters (Kaikadi *et al.* 2006). Based on the above results *churpe*-balls and *churpe*-strips containing 15% apricot powder and 09% spinach powder, respectively, were found to be most acceptable by the panelists. However, overall acceptability scores of the all the supplemented products were still in the category of 'like'.

Titratable acidity of *churpe* products are presented in Table 6. The products demonstrated an excellent improvement in it with the rise in the levels of supplements. The mean titratable acidity increased from 6.24% to 6.74% at 5% and 20% levels of apricot

Table 5. Effect of supplementation and storage on overall acceptability of value-added *churpe* products (balls and strips)

Supplementation level (%)	Storage period (days)					
	0	90	180	270	360	Mean
Control (Cottage cheese)	06.30 ± 0.32	06.30 ± 0.13	06.00 ± 0.27	05.90 ± 0.18	05.40 ± 0.27	05.90 ± 0.23
CC:AP:GS						
90:00:10	07.40 ± 0.21	07.20 ± 0.21	06.90 ± 0.29	06.30 ± 0.15	06.00 ± 0.17	06.70 ± 0.20
85:05:10	07.60 ± 0.30	07.10 ± 0.19	06.70 ± 0.21	06.40 ± 0.26	06.10 ± 0.19	06.70 ± 0.23
80:10:10	07.90 ± 0.14	07.70 ± 0.18	07.40 ± 0.16	07.20 ± 0.20	07.20 ± 0.29	07.40 ± 0.19
75:15:10	08.50 ± 0.19	08.50 ± 0.28	08.20 ± 0.20	07.80 ± 0.16	07.60 ± 0.12	08.10 ± 0.19
70:20:10	07.20 ± 0.13	06.00 ± 0.37	05.80 ± 0.18	05.40 ± 0.11	05.00 ± 0.10	05.80 ± 0.17
Mean	07.50 ± 0.19	07.10 ± 0.24	06.80 ± 0.20	06.40 ± 0.17	06.10 ± 0.17	06.80 ± 0.19
CD (P < 0.05)	Supplementation = 0.14		Storage = 0.15		Supplementation x Storage = 0.35	
CC:SP						
97:03	06.10 ± 0.18	05.70 ± 0.30	05.30 ± 0.29	05.10 ± 0.21	04.50 ± 0.33	05.30 ± 0.26
94:06	07.00 ± 0.17	06.60 ± 0.12	06.10 ± 0.24	05.80 ± 0.13	05.50 ± 0.34	06.20 ± 0.20
91:09	07.70 ± 0.23	07.40 ± 0.35	06.90 ± 0.10	06.60 ± 0.14	06.20 ± 0.30	06.90 ± 0.22
88:12	06.90 ± 0.18	06.60 ± 0.29	06.40 ± 0.19	06.00 ± 0.36	06.00 ± 0.29	06.30 ± 0.26
Mean	06.90 ± 0.19	06.50 ± 0.26	06.10 ± 0.20	05.80 ± 0.21	05.50 ± 0.31	06.20 ± 0.23
CD (P < 0.05)	Supplementation = 0.18		Storage = 0.18		Supplementation x Storage = NS	

CC = Cottage cheese, AP = Apricot Powder, GS = Ground sugar, SP = Spinach powder, n = 3

Table 6. Effect of supplementation and storage on titratable acidity of value-added *churpe* products (balls and strips)

Supplementation level (%)	Storage period (days)					
	0	90	180	270	360	Mean
Control (Cottage cheese)	06.27 ± 0.11	06.73 ± 0.14	06.52 ± 0.11	06.49 ± 0.16	06.32 ± 0.05	06.46 ± 0.11
CC:AP:GS						
90:00:10	05.05 ± 0.17	05.46 ± 0.16	05.33 ± 0.25	05.12 ± 0.23	05.02 ± 0.27	05.19 ± 0.21
85:05:10	05.82 ± 0.15	05.97 ± 0.19	06.33 ± 0.15	06.47 ± 0.17	06.61 ± 0.03	06.24 ± 0.13
80:10:10	06.41 ± 0.08	06.01 ± 0.07	06.12 ± 0.18	06.36 ± 0.03	06.73 ± 0.04	06.32 ± 0.08
75:15:10	06.47 ± 0.26	06.62 ± 0.25	06.80 ± 0.06	06.85 ± 0.06	06.96 ± 0.06	06.74 ± 0.13
70:20:10	06.63 ± 0.12	06.64 ± 0.06	06.70 ± 0.13	06.82 ± 0.16	06.95 ± 0.18	06.74 ± 0.13
Mean	06.07 ± 0.15	06.14 ± 0.14	06.25 ± 0.16	06.32 ± 0.13	06.45 ± 0.11	06.24 ± 0.14
CD (P < 0.05)	Supplementation = 0.11		Storage = 0.10		Supplementation x Storage = 0.25	
CC:SP						
97:03	06.06 ± 0.09	06.08 ± 0.04	06.18 ± 0.03	06.46 ± 0.31	06.63 ± 0.06	06.28 ± 0.16
94:06	06.30 ± 0.05	06.44 ± 0.04	06.76 ± 0.28	06.81 ± 0.21	06.88 ± 0.35	06.63 ± 0.17
91:09	06.48 ± 0.19	06.81 ± 0.19	06.84 ± 0.09	06.92 ± 0.19	06.96 ± 0.24	06.80 ± 0.20
88:12	06.60 ± 0.07	06.66 ± 0.14	06.67 ± 0.14	06.89 ± 0.09	06.96 ± 0.24	06.75 ± 0.12
Mean	06.36 ± 0.10	06.49 ± 0.10	06.61 ± 0.20	06.77 ± 0.20	06.85 ± 0.22	06.61 ± 0.16
CD (P < 0.05)	Supplementation = 0.12		Storage = 0.12		Supplementation x Storage = 0.27	

CC = Cottage cheese, AP = Apricot Powder, GS = Ground sugar, SP = Spinach powder, n = 3

Table 7. Effect of supplementation and storage on pH of value-added *churpe* products (balls and strips)

Supplementation level (%)	Storage period (days)					
	0	90	180	270	360	Mean
Control (Cottage cheese)	05.17 ± 0.23	05.15 ± 0.25	05.07 ± 0.08	04.76 ± 0.15	04.64 ± 0.09	04.95 ± 0.16
CC:AP:GS						
90:00:10	05.26 ± 0.26	05.19 ± 0.14	05.14 ± 0.05	05.05 ± 0.07	04.75 ± 0.18	05.07 ± 0.14
85:05:10	05.11 ± 0.38	04.74 ± 0.08	04.58 ± 0.19	04.44 ± 0.09	04.47 ± 0.14	04.66 ± 0.17
80:10:10	04.78 ± 0.05	04.43 ± 0.11	04.31 ± 0.24	04.94 ± 0.18	04.11 ± 0.08	04.51 ± 0.13
75:15:10	04.52 ± 0.07	04.37 ± 0.21	04.19 ± 0.28	04.71 ± 0.29	04.51 ± 0.04	04.46 ± 0.17
70:20:10	04.02 ± 0.13	04.73 ± 0.04	04.56 ± 0.04	04.25 ± 0.16	04.07 ± 0.16	04.32 ± 0.10
Mean	04.76 ± 0.17	04.69 ± 0.11	04.55 ± 0.05	04.67 ± 0.15	04.38 ± 0.12	04.60 ± 0.14
CD (P < 0.05)	Supplementation = 0.12		Storage = 0.11		Supplementation x Storage = 0.28	
CC:SP						
97:03	04.93 ± 0.04	04.76 ± 0.14	04.73 ± 0.20	04.60 ± 0.15	04.54 ± 0.28	04.71 ± 0.16
94:06	04.68 ± 0.18	04.53 ± 0.19	04.49 ± 0.06	04.46 ± 0.04	04.37 ± 0.24	04.50 ± 0.14
91:09	04.29 ± 0.19	04.29 ± 0.06	04.28 ± 0.17	04.24 ± 0.06	04.12 ± 0.16	04.24 ± 0.12
88:12	04.18 ± 0.28	04.10 ± 0.15	04.10 ± 0.12	04.03 ± 0.25	04.00 ± 0.07	04.08 ± 0.17
Mean	04.52 ± 0.17	04.42 ± 0.13	04.40 ± 0.13	04.33 ± 0.12	04.25 ± 0.18	04.38 ± 0.15
CD (P < 0.05)	Supplementation = 0.12		Storage = 0.12		Supplementation x Storage = NS	

CC = Cottage cheese, AP = Apricot Powder, GS = Ground sugar, SP = Spinach powder, n = 3

powder, respectively and the same increased from 6.28% to 6.75% at 3% and 12% levels of spinach powder. The increased titratable acidity of the supplemented *churpe* products seems to be, due to the direct effect of the higher amount titratable acids present in apricot and spinach. The acetic acid, citric acid, malic acid and tartaric acid are the main organic acids that are present in apricot (Ghnimi *et al*., 2018) thus conferring high acid content to the product. Similar results were reported for probiotic yogurt supplemented with passion

fruit or pineapple peel powders (Espirito *et al*., 2012; Sah *et al*., 2016). The addition of apricot to fruit-cheese bar, spinach powder to UF-soft cheese and *Moringa oleifera* leaves powder to soft cheese enhanced acid development (Jabeen *et al*., 2020; El-Sayed, 2020; Hassan *et al*., 2017). Storage led to increase in titratable acidity, from 6.07 to 6.45 in balls and from 6.36 to 6.85 in strips. Similar results were reported by El-Sayed (2020) in soft cheese.

It was observed that pH of *churpe*-balls decreased with increase in the level of fortification of apricot powder in fresh cheese (Table 7). Control *churpe* had mean pH of 4.95 which got reduced significantly ($P < 0.05$) from 4.66 to 4.32 at 05% to 20% levels of apricot powder. This effect may be attributed to higher acid contents in apricot. The addition of apricot to fruit-cheese energy bar significantly reduced the pH (Jabeen *et al*., 2020). The mean pH decreased significantly ($P < 0.05$) upon increasing the fortification levels of spinach powder in *churpe*-strips. It was found that, at 03% level of supplementation, the mean pH was 4.71 which got declined up to 4.08 at 12%. This could be contributed to higher acidity of spinach powder. Similar results were also reported by El-Sayed (2020) and Hassan *et al*. (2017) in cheeses supplemented with spinach powder and *Moringa oleifera* leaves. Storage period of 120 days resulted in decline of pH of both products i.e. from 4.76 to 4.38 in balls and 4.52 to 4.25 in strips. The pH values of parsley added cheeses were also reported to be decreased gradually during the storage period. (El-Taweel *et al.* 2017). pH of spinach powder supplemented UF-soft cheese decreased during 4 weeks of storage (El-Sayed, 2020). The pH of whey protein concentrate decreased significantly ($P < 0.05$) after 18 months (Tunick *et al*., 2015) and up to 2 months (Rathour *et al*., 2017) of storage. Decrease in pH during storage was reported by Coulter *et al*. (1948) in dried whole milk and suggested that amino group binding with lactose in Millard reaction as the cause which was also supported by (Kehrberg and Johnson, 1975). Free fatty acid formation presumably contributed to its pH decrease. The pH value of the samples might also be decreased during storage due to the lactic acid produced by the lactic acid bacteria (Yildiz and Ozcan, 2018).

Conclusion

The traditional *churpe* of Ladakh when incorporated with apricot and spinach become more nutritious having health benefits. Upon comparing the various levels of additives, it was found that 15% supplementation of apricot in *churpe*-balls and 9% supplementation of spinach in *churpe*-strips provide the best combinations in terms of organoleptic scores. The sensory attributes of the value- added products remain stable with minor changes during the storage period of 120 days.

Conflict of interest

The authors declare that they have no conflict of interest.

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Comparative efficacy and validation of different diagnostic methods in detection of subclinical mastitis in farms of Bundelkhand

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Abstract

A cross-sectional study was undertaken to validate the comparative efficacy of different diagnostic tests commonly used for the detection of subclinical mastitis. The study was designed to determine the specificity, sensitivity, and accuracy of common tests viz. California Mastitis Test (CMT), Indicator Paper Method (BTB), Somatic Cell Count (SCC), and Electrical Conductivity (EC) with bacterial culture as a standard, in parts of Bundelkhand region. Of the 411 samples, 295 were found to be positive for subclinical mastitis on screening with either of the 4 methods routinely used for the diagnosis of subclinical mastitis (SCM). The remaining 120 milk samples were negative for SCM with all methods used. Individual test-wise percentage prevalence was 26.28, 44.53, 30.41, and 41.85% respectively with CMT, EC, BTB, and SCC, respectively. On comparing the results with the culture test, the present study confirmed the superiority of SCC based method for the detection of subclinical mastitis. Correlating the EC results with SCC values avoids false results with maximum accuracy.

Keywords: *Subclinical mastitis, mastitis detection, validation, CMT, SCC*

Mastitis refers to inflammation of the mammary gland due to infection during which, the tissues and ducts in the secretary system of the glands become damaged by persistent and opportunistic infection of microorganisms. The disease leads to increased economic burden because of the associated costs in preventive measures, management and related changes in qualitative as well as quantitative terms due to degradation of milk quality. Bovines are widely used for milk production throughout the world and bovine mastitis is a major factor affecting milk production. Of the two forms of mastitis *viz.*, clinical and subclinical, subclinical mastitis (SCM) is of greater concern due to the invisible health effects on the animals, where scientific monitoring of animals, as well as farmers is quite significant in enforcing control measures. Adoptability of tests as well as their sensitivity and accuracy are important factors to be taken into consideration for the correct, rapid and real-time diagnosis of the disease to determine the course of treatment.

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Unfortunately, 20-25 per cent of the burden of cases occur due to subclinical mastitis and is neglected due to its invisible nature, inviting delayed treatment in the present-day animal husbandry sector. This burden might be much higher in developing countries like India due to the lack of data from the hinterlands and local farms. Electrical conductivity of milk and counting the somatic cells were considered important strategies to detect subclinical mastitis in previous studies (Hegde *et al*., 2013). The authors also advised fine-tuning in SCC and EC so as to limit the higher probability of positive correlation between the coagulase-negative staphylococci (CoNS) and somatic cell count. The correct, specific diagnostic methods for different agroclimatic regions may have greater implications in identification, management, and clinical course recommendation to control the subclinical mastitis.

As per recommendations made by the International Dairy Federation, Somatic Cell Count is the recommended test with standard as bacterial culture (Reddy *et al*., 2014), but it is seen that varying results have been obtained in previous studies and it is important to determine the validity for the efficacy of the tests concerning different agro-climatic regions to establish the efficacy of field tests so these could be used as a choice for rapid on-site delivery of testing services. Considering this approach, a study was designed to assess the comparative efficacy of different detection tests in parts of Bundelkhand.

Materials and methods

Milk samples were collected from three districts of the Bundelkhand region *i.e.* Jhansi, Jalaun, and Lalitpur. A preliminary survey for subclinical mastitis based on history and occurrence was conducted in Jhansi, Lalitpur, and Jalaun Districts. Villages were selected randomly. Fresh milk samples were directly collected quarter-wise, except in a few cases, where composite milk samples had to be collected owing to difficulty in animal handling. During sample collection, all sanitary measures were considered as per the National Mastitis Council, USA. Prior cleaning of the udder with water followed by 70 per cent alcohol was done before collecting milk samples. After 5 minutes, 10 ml of milk sample was directly drawn into horizontally tilted falcon tubes to avoid contamination due to skin shedding. Each sample was assigned with a code number for the owner of the animals. Milk sample for California mastitis test was directly drawn into the cups of CMT paddle to check mastitis status on field. A thermocol box filled with ice packs was used to transport the samples. Samples were processed immediately for bacteriology, SCC count and EC. The remaining milk sample was stored at -80°C. All culture media and supplements were purchased from Himedia laboratories, Mumbai and reagents used were of analytical and molecular grade.

Screening of subclinical mastitis in the milk samples

SCC, CMT, EC, and BTB Strip-based techniques were used to screen subclinical mastitis in all the collected milk samples. The procedure described by Galdhar *et al.* (2004) was followed in the screening of bovine milk samples for subclinical mastitis. CMT working reagent is composed of anionic surface active compounds (Teepol 0.5% (v/v) + NaOH 1.5% (w/v)\) mixed thoroughly with an indicator dye, bromothymol blue 0.01% (w/v). The results of CMT was interpreted as in Table 1. The immediate reaction leads to the formation of gel and precipitation in positive samples. The appearance of milk samples that tested positive for mastitis was greenish due to alkalinity while the increased number of leucocytes was responsible for gel formation (Table 1).

Vliegher *et al.* (2012) showed the variability in the type of infections for the quarter. Sample collected from each quarter was considered as individual samples. The calculated sample size was considered for the study following formula devised by Thrusfeild (2005).

$$n = \frac{z^{2*} P_{\text{exp}} \ (1 - P_{\text{exp}})}{d^2},$$

n=sample size needed z= α value at 95 per cent confidence interval=1.96 Pexp= prevalence expected d= precision desired.

Table 1. Interpretation Chart for California Mastitis Test in correlation with SCC count

Visible reaction	CMT Score given	Interpretation	SCC/ml
No change in milk	1	Negative	<100000
Light precipitation	2	Trace	b/w 1-200000
Clear precipitation, No gel formation	3	Weak +Ve	b/w 2-400000
Solution thickens and gel formation	4	+Ve	b/w 4-500000
A great increase in viscosity and strong gel	5	Strong +Ve	>500000

Procured milk samples were undertaken for the estimation of electrical conductivity with a handheld instrument (Jyothi Scientific Industries, Gwalior). EC≥6.5mS/cm was considered to mark the sample positive for subclinical mastitis.

The freshly collected milk samples were processed to estimate the number of somatic cell count by direct microscopy method. Direct screening of milk samples to determine mastitis status by counting somatic cells was done with Newmann's staining with microscopy. The procedure described by Prescott and Breed (1910) in general and used by Schalm *et al*. (1971) was followed. Microscopic field diameter seen through oil immersion lens was measured up to two decimal points using stage micrometer slide ruler in 0.1 to 0.01 mm. Formula πr^2 was used to calculate the area of the field. Milk pH was determined with a digital pH meter supplied by Jyoti Scientific, Gwalior. Standard buffer solutions of pH 4, 7.2, and 9.2 were used to calibrate the pH meter each time before use. The somatic cell count includes accounting for different inflammatory cells in stained milk smear, which includes polymorpho-nuclear (PMN) cells (including neutrophils, lymphocytes, macrophages). The secretory glandular desquamated epithelial cells were also considered in SCC counting. The somatic cell count >5 lakhs/ml, conventional criteria was considered to declare the milk samples as positive for subclinical mastitis.

Bacteriological culture of milk

Bacteriology related wet lab work, biochemical characterization, and primary bacteriological diagnosis were carried out at the Department of Biotechnology, Bundelkhand University Jhansi by culturing milk samples on different generalized and selective bacteriological media according to previous studies described by Collee *et al.* (1996), with local modifications to adjust pH and solidification. In accordance to Ericsson *et al*. (2009), Samples positive for at least 3 cfu/ml were considered as bacteriologically positive for all bacterial genera. But the growth of a single colony was considered positive in the case of Staphylococci.

The purity and growth quantity of each agar plate was determined and categorized as abundant if, >50 cfu/ml, moderate 10-50 cfu/ml, and mild, if it was less than 10 cfu/ml. Further, bacteria were classified by colony morphology and haemolysis characteristics. The further classification was done by biochemical characterization in accordance with Bergey's manual of determinative bacteriology based on specific genera (Holt *et al*., 1994). Criteria used in classification were based on colony morphology following Hogan (1999). Media plates found positive for three or more different pathogens were considered as mixed cultures.

Results and discussion

Data for samples and the number of samples found positive for subclinical mastitis with different tests in different districts and herds are presented in Table 2. Further, Table 3 reveals the comparative statistical significance of the results obtained with these four tests by using the analysis of variance.

California Mastitis Test

Out of total of 411 milk samples, 108 (26.27 per cent) samples were found positive for subclinical mastitis. Recorded teat-wise prevalence of subclinical mastitis was 25.97, 30.77 and 21.05 per cents in Jhansi, Jalaun, and Lalitpur district, respectively.

Table 2. Prevalence of subclinical mastitis (SCM) determined with different diagnostic tests

Sr. No.	Site	No. of samples collected	Number of samples found positive			
			CMT	EC	BTB	SCC
Jhansi						
1.	Site-1	49	15	20	08	22
2.	Site 2	54	13	23	16	24
3.	Site 3	51	12	26	12	28
	Total	154	40	69	36	74
Percentage		-----	25.97	44.81	23.38	48.05
Jalaun						
4.	Site 1	50	14	21	12	25
5.	Site 2	40	12	22	20	15
6.	Site 3	53	18	17	21	17
	Total	143	44	60	53	57
Percentage		-----	30.77	41.96	37.06	39.86
Lalitpur						
7.	Site 1	30	08	18	16	13
8.	Site 2	40	07	14	12	12
9.	Site 3	44	09	22	08	16
	Total	114	24	54	36	41
Percentage		-----	21.05	47.36	31.58	35.96
Grand total		411	108	183	125	172
Percentage		-----	26.28	44.53	30.41	41.85

Table 3. Comparison between the results obtained with different tests

N	CMT	EC	BTB	SCC	Total
	9	9	9	9	36
∑X	108	183	125	172	588
Mean	12	20.3333	13.8889	19.1111	16.333
$\sum X^2$	1396	3823	1913	3552	10684
Std.Dev.	3.5355	3.5707	4.7022	5.7542	5.5549

Table of significance

Source	*SS*	*df*	*MS*	
Between-treatments	436.2222	3	145.4074	*F* = 7.22771
Within-treatments	643.7778	32	20.1181	
Total	1080	35		

The *f*-ratio value is 7.22771. The *p*-value is .000777. The result is significant at $p<.05$.

Electrical conductivity (EC) measurement

Samples were categorised into two groups based on EC values ≥6.5mS and <6.5mS. EC value was found ≥ 6.5mS for 183 (44.53%) samples and <6.5 mS for remaining 228 (55.47%) samples. An EC value greater than 6.5mS was considered as standard to declare the sample as positive for subclinical mastitis. The recorded prevalence of subclinical mastitis was 44.53 per cent with this method.

Indicator paper method (Bromothymol Blue strip test)

Out of 411 samples screened for SCM, 30.41 per cent (125) milk samples were found positive for subclinical mastitis with BTB strip test.

Somatic cell count (SCC) estimation

The number of cases of subclinical

mastitis was more in semi-urban areas as compared to rural areas. Based on total counts of somatic cells, milk samples were divided into 4 different groups *i.e*. 0-1, 1-2, 2-5, and >5 lakhs cells/ml. It is recorded that 380 out of 411 milk samples were found to show the countable somatic cells in the range of 0-5 lakhs. Individually, 89,68, 82 and 172 samples revealed SCC value in range of 0-1, 1-2, 2-5 and >5 lakhs/ml, respectively. Somatic cells could not be determined in 31 samples, and these were considered in the group 0-1 lakhs/ml. Thus, conventional criteria showed the 41.85 per cent prevalence of subclinical mastitis.

Overall, 295 out of 411 samples were found to be positive for subclinical mastitis on screening with either of the four methods of routine use in the diagnosis of subclinical mastitis. The remaining 116 milk samples were negative for SCM with all methods used. This data revealed an overall prevalence of 71.78 per cent on considering all tests as valid.

The normal appearance of milk and absence of visible signs in the mammary gland tissue is the major barrier in the early detection of subclinical mastitis (Mishra *et al*., 2018). SCM creates a reservoir of microorganisms that act as a source of infection to the other individuals and help in the preponderance of clinical mastitis (Thompson *et al*., 2014). More than 137 organisms belonging to different classes and taxa have been identified as pathogens of bovine subclinical mastitis, including bacteria, viruses, fungi, algae, and mycoplasma (Watts, 1988). *Staphylococcus aureus* has been considered as the major causative agent (Verma *et al.,* 2017) but coagulase-negative Staphylococci have been reported by many authors in different countries as the most prevalent pathogen in bovine subclinical mastitis e.g. Poland, Iran, and India (Sztachanska *et al*., 2016; Chavoshi and Husaini, 2012; Hegde *et al.,* 2013). So the objectives designed were in concurrence with the studies with a focus on the Bundelkhand region. A similar pattern of dominance of Coagulase-negative staphylococci was also found in our study. \ As most of these organisms are commensals in human being, studies on the impact of single species on bovine udder health are possible only after the accurate identification of causatives agents (Zadocks and John, 2011). Data presented in Table 2 reveals the overall prevalence of SCM in the selected region with 4 different tests *viz*., CMT, EC, BTB strip and SCC with values 26.28, 44.53, 30.41 and 41.85 per cent, respectively. Reliability of method based on SCC for the determination of prevalence of subclinical mastitis is established in previous studies (Hegde *et al*., 2013). The international dairy federation also recommends the SCC and bacterial culture-based diagnostic test to detect mastitis (Anonymous, 1971). The cut-off value of SCC count to consider the animal positive for SCM varies from country to country *viz*., $4x10^5$ cells/ml in New Zealand and Australia, $5x10^5$ cells/ml in Canada, $2x10^5$ cells/ml in Sweden, and $7.5x10^5$ cells/ml in the USA. However, no standard cut-off is prescribed in India, so the cut-off value considered was $5x10^5$. But National Mastitis Council, USA prescribes all the above 4 tests for the detection of mastitis. So, it is considered that no single test can detect SCM with 100 per cent accuracy. Therefore, we have used all four tests for prevalence studies. Based on somatic cell count, the highest prevalence of SCM was found in Jhansi district with 48.05 per cent followed by Jalaun and Lalitpur where prevalence was 39.86 and 35.96 per cent, respectively. Previously, a 42.85 per cent prevalence of subclinical mastitis was reported in Bidar, Karnataka (Ambika *et al.,* 2021) and 34.9-46.00 per cent in Dharwad, Karnataka (Mahantesh *et al*., 2014). The prevalence of bovine subclinical mastitis in India and worldwide, was reported to be in the range of 30-50 per cent (Sanotharan *et al*., 2016; Mpatswenumugabo *et al*., 2017; Said *et al*., 2018).

EC test for the detection of subclinical mastitis is also recommended by the International Dairy Federation. Based on the EC test, the prevalence was found 44.81, 41.96, and 47.36 per cent, respectively for Jhansi, Jalaun, and Lalitpur districts. The recorded range was in accordance with SCC but for the Lalitpur district, it was very high in comparison to SCC-based prevalence results. One of the reasons behind this disparity may be the low sample size from the Lalitpur district. Findings are in close agreement with Shabaz *et al*. (2020) but lower than Jinu and Singh,

(2020). The opening of alveolar junctions and increase in permeability of capillaries due to bacterial infections is responsible for higher electrical conductivity followed by an increase of secretion of high Na+, K+, and Cl- into the extracellular fluid. Ultimately level of these ions is also increased in the milk of the infected glands. EC test thus shows this increased ion content in the milk samples of infected quarters (Paudyal *et al*., 2020). A significant difference was observed within the group ($p<0.05$)

CMT test was performed in accordance with guidelines established by National Mastitis Council, highest SCM prevalence was recorded in Jalaun district (30.77%) followed by 25.97 and 21.05 per cent, respectively for Jhansi and Lalitpur. Our results are in agreement with studies done by different authors in various parts of the country (Senthilkumar *et al*., 2020; Karabasanavar *et al.* 2019; Swami *et al.,* 2017). Results produced with CMT may differ from SCC-based prevalence determination (Iraguha *et al*. 2017). They reported lower prevalence for a selected population with CMT test in comparison to the SCC-based method in a similar study on sensitivity comparison. BTB strip test-based diagnostics reported the highest prevalence of 37.06 per cent in Jalaun followed by 31.58 and 23.38 per cent, respectively for Lalitpur and Jhansi. Prevalence of mastitis based on CFT differed from the cross-sectional study done in 2018 in Jhansi with partial and low sample sizes (Singh and Kumar, 2018). Table 3 shows the significant difference in prevalence determined by all four tests ($p<0.05$). So none of the single methods can be considered as reliable for the determination of prevalence of subclinical mastitis, But SCC was the only method that is used as confirmatory evidence in previous studies (Chakraborty *et al.,* 2019; Iraguha *et al*., 2017). So in the present investigation, SCC results were used to determine the ultimate prevalence of subclinical mastitis in the selected region which differed with the other 3 tests *viz.* CMT, BTB and EC, significantly ($p<0.05$). ANOVA revealed the significant difference in prevalence reported from different sites and by different methods also.

Bacterial isolation from milk samples

Studies on bacteriology of milk samples completed by microscopy, total viable count on nutrient agar and culturing on Cystine Lactose electrolyte-deficient Agar (CLED) to differentiate contaminants, mannitol salt agar (MS) as selective for gram positive bacteria, haemolysis studies on blood agar. Different species of bacteria were categorized into separate groups on the basis of microscopic character sticks and colony morphology. Collected milk samples were inoculated to the McConkey agar and Blood agar base enriched with 7% defibrinated sheep blood followed by aerobic incubation for 24 to 48 hours at 37^0C with modifications to adjust pH, solidification. Although, 41.58 per cent of samples were identified as positive for subclinical mastitis, all the samples were subjected to bacterial isolation. Total 365 isolates belonging to *CoNS*, *S. aureus*, *Streptococcus sp*., *Bacillus*, *Corynebacterium* and *E.coli* based on microscopic and cultural characteristics were identified. Coagulase-negative staphylococci (CoNS) and *Staphylococcus aureus* were found to correlate with the pattern of somatic cell count. The largest numbers of staphylococcal isolates were recovered from the samples showing SCC > 5 lakhs/ml. Three hundred and sixty-five (88.8%) out of 411, samples were found bacteriologically positive (Table 4). The bacterial counts on different media are presented in Table 5.

Table 4. Bacterial isolation from milk samples

Place	Collected number of samples	CoNS	CoPS	Total number of Staphylococci
Jhansi	154	49	19	68
Jalaun	143	48	12	60
Lalitpur	114	48	13	61
Total (Subclinical)	172	125	44	169
Clinical	15	02	Nil	02

Table 5. Total bacterial counts on different media in cfu/ml.

SCM Status	Source	Nutrient agar	CLED Agar	McKonkey Agar	MS Agar
SCM +Ve	Buffalo	$2.14x10^5$	$9.55x10^4$	$8.4x10^3$	$1.65x10^4$
	Cow	$1.24x10^5$	$7.57x10^4$	$6.0x10^3$	$1.14x10^4$
SCM -Ve	Buffalo	$7.4x10^4$	$8.22x10^4$	$8.6x10^3$	$1.65x10^4$
	Cow	$6.5x10^4$	$5.25x10^4$	$1.9x10^3$	$4.8x10^3$

A total of 365 bacterial isolates were identified by biochemical microbial culture followed by biochemical identification. Standard biochemical tests used for identification in the study are in accordance with Bergey's Manual of Determinative Microbiology as adopted by Collee *et al.,* 1996. Centrifugation of all the collected milk samples was carried out to increase the probability of bacterial detection as recommended previously (Lima *et al*., 2018; Jinu and Singh, 2020). Bacteria could be isolated from 41.8 per cent of samples, which was in the alignment of the results obtained through somatic cell count followed by EC Test. Mixed infections were found very common as 220 milk samples were found to infect with more than one bacterial group.

Conclusion

Present study confirms the superiority of SCC based method for the detection of subclinical mastitis. Correlating the EC results with SCC values may avoid false results with maximum accuracy. SCC was found most reliable diagnostic test but from point of ease of doing on-field detection of SCM. EC is recommended followed by confirmation through SCC as evident from the findings of this study. However, considering the zone to zone variations, it is recommended to extend the study to different climatic zones for specific recommendations on grouping of the tests.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Characteristics of the leachate produced during nutrient recycling of food and poultry slaughter wastes by fly larvae

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Abstract

Biowaste management is a pertinent problem and nutrient recycling utilizing fly larvae is an emerging solution. The leachate produced during biowaste management has potential implications for the environment but studies on this issue are scanty. This study was conducted to assess the characteristics of leachate produced during the bioconversion of food (BW-I) and poultry slaughter waste (BW-II) by natural fly larval activity. The results showed that total volumes of 2.2 L and 1.1 L leachate were produced for BW-I and II respectively. The highest pH in BW-I leachate was 7.43 ± 0.01 and the lowest pH in BW-II was 3.30 ± 0.12. The highest levels of BOD in BW-I and II were 36733.33 ± 430.63 mg/l and 2800.000 ± 999.50 mg/l. The highest level of COD in BW-I was 52575.000 ± 1076.86mg/l while in BW-II it was 4316.67 ± 790.45 mg/l. The high BOD and COD values of the leachate indicated that they needed to be pretreated before being released into the environment.

Keywords *: Food waste, poultry slaughter waste, leachate, BOD, COD*

Management of biowaste is a pertinent problem today. Several decentralized management options are available to overcome this issue, but the impact of these options on the micro environment, fauna and flora have not been fully assessed. Decomposition of solid biowastes, result in the release of part of the moisture as leachate endowed with several potential environmental implications. Leachate has been described as the liquid that is produced by the degradation of

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waste and is stored along with the percolation of rainwater (Hernández-García *et al.,* 2019). The irrational management of solid waste results in a significant wastage of resources and this is further precipitated by the release of a large amount of polluted leachate which has a significant effect on the environment (Feng *et al*., 2019). Reduced space for waste disposal due to the increased human population was a cause for contamination of water resources (Latha *et al.,* 2003). The pH of leachate, BOD and COD could have implications on the soil and immediate surroundings of where it is generated or drained. Food waste recycling has been found to produce leachate of acidic nature due to fermentative reactions. Solid biowastes with high moisture content produce a significant quantity of leachate and hence, appropriate knowledge of the characteristics of the leachate produced is essential to initiate measures to reduce environmental and public health issues associated with bio waste handling and management. Nutrient recycling utilizing fly larvae is an emerging technology for biowaste management; this technology helps to reduce the loop of recycling to a circular economy. Hence, the present study was taken up to analyse the characteristics of the leachate released during the bio-conversion of food and poultry slaughter wastes by fly larvae.

Materials and methods

The study was conducted at the Eco-farm unit under the Department of Livestock Production Management, College of Veterinary and Animal Sciences, Mannuthy, Kerala Veterinary and Animal Sciences University, Thrissur, Kerala. The study was conducted for a period of one year from November 2017 to December 2018. The study period was divided into three seasons as categorised by Joseph (2011) namely;

(i) Summer months (February-May) - S-I
(ii) Monsoon months (June-September) - S-II
(iii)Post-monsoon months (October-January) - S-III

Experimental setting

The poultry slaughter waste (BW-I) was collected locally from Mannuthy market, and this included the digestive system and associated wastes, excluding feather, head and feet**.** Food waste (BW-II) consisted of household and hostel food waste collected from Mannuthy campus. Five kilograms from each source were placed in 25 L bins with six replicates and a total of 12 bins were set. Containers (five liter) were kept below the bins to collect the leachate and further studies on the characteristics of the same. The experiment was conducted for 30 days during each season. The total period of 30 days was divided into five phases of six days each. The five phases were designated with identifying numbers from P-I to P-V. Flies were allowed to arrive naturally and ovulate on the biowastes and their larvae were allowed to convert the biowaste.

Physicochemical changes of the leachate

The physical and chemical changes in the leachate including the volume and pH, were recorded daily. The volume of the leachate was noted using a 1 L graduated measuring jar (Sarpong *et al.,* 2018). The pH of the leachate was recorded using PCSTestr 35 (Eutech, Thermo Scientific) (Oviedo-Ocaña *et al*., 2015).

Biochemical Oxygen Demand (BOD)

The samples of leachate that were collected were subjected to analysis for biochemical oxygen demand (BOD) (APHA, 2012), at weekly intervals.

*Chemical Oxygen Demand (COD***)**

Samples from the collected leachate were taken for estimation of chemical oxygen demand (COD) at weekly intervals. COD was done using the COD Cell Test kit Spectroquant 1.01797.001 (Merck) with a measuring range of 5000 to 90000 mg/L COD (Marcheggiani *et al.,* 2019).

Results and discussion

Physicochemical changes in the leachate

The BW-I had an initial moisture content that ranged from 73 to 76 per cent, and finally reduced to a level of 41 to 48 per cent. The BW-II had an initial moisture content that

BW-I leachate BW-II leachate

Fig. 1. Colour and nature of leachate from BW-I and II

ranged between 75 and 79 per cent, which reduced to a final moisture level that ranged from 70 to 76 per cent. The loss in moisture was a factor that mainly contributed to the formation of leachate.

The volume of leachate produced is given in Table 1. Leachate production occurred only during the first two phases. The BW-I leachate had pungent smell with a fat layer, and was dark red to dark brown in colour (Fig. 1). The BW-II leachate was cream or light brown in colour with an alcoholic smell and a watery consistency. The highest total volume of leachate produced was 2.2 L in BW-I in S-I, while for BW-II, it was 1.1 L in S-III. The maximum volume of leachate produced from BW-I (1.99 ± 0.045) was observed during P-I of S-II and from BW-V (0.52 ±0.03) during P-I of S-III. This is in agreement with the report of USDA (2004), where it was reported that more than 50 per cent of the available volume of fluid would leach out in the first week of degradation. Ghanem *et al.* (2001) reported that the maximum leachate production from food waste occurred by 10 days, which was in agreement with our findings. Means having same superscripts (small letter a-e, h-l, v-z within columns, capital letters A-E, H-L, V-Z within rows) did not differ significantly at 5 per cent level. The pH changes observed during the experimental period in all the leachates are given in Table 2. The highest pH in BW-I leachate (7.43 ± 0.01) was observed in P-I of S-II, similar to that reported for poultry slaughter waste water by Rajakumar *et al.* (2011), which was 7 to 7.6. But the value reported by Chavez *et al.* (2005) was 6.6 which was lower than that obtained in the present study. The lowest pH of BW-II leachate was 3.30 ± 0.12 and it ranged between 3.3 and 4.65, which was lower than that reported by Wang *et al.* (2013) and Zamri *et al.* (2016) for landfill leachate for which the pH was 8-8.1. But the value was closer to that reported by Sall *et al.* (2019) in studies on vegetable and fruit compost leachate (5.4). This may be because, in landfill leachate, the leachate had already undergone a maturation phase with a stabilisation of pH, whereas the leachate from vegetable and fruit compost produced an acidic leachate.

Table 1. Average volume (L) of poultry slaughter waste and food waste leachate

Season	Phase	Poultry slaughter waste	Food waste
Summer	I	1.89 ± 0.04^{bB}	0.41 ± 0.04^{bA}
	II	0.11 ± 0.02^{aA}	0.12 ± 0.02^{aA}
Monsoon	I	1.99 ± 0.05^{il}	0.39 ± 0.05^{iH}
	II	0.21 ± 0.01^{hH}	0.19 ± 0.01^{hH}
Post-monsoon	I	1.73 ± 0.03^{wW}	0.52 ± 0.03^{wV}
	II	0.31 ± 0.04^{vV}	0.19 ± 0.04^{vV}

Table 2. pH of poultry slaughter waste and food waste leachate

Season	Phase	BW-I	BW-II
Summer	I	7.00 ± 0.18^{bB}	3.30 ± 0.12^{aA}
	II	6.17 ± 0.03^{aA}	4.08 ± 0.02^{bB}
Monsoon	I	7.43 ± 0.01^{hl}	3.71 ± 0.01^{hH}
	II	7.34 ± 0.06^{hl}	4.65 ± 0.06^{iH}
Post-monsoon	I	7.02 ± 0.03^{vV}	3.78 ± 0.03^{wW}
	II	7.11 ± 0.02^{wV}	3.53 ± 0.02^{vW}

Means having same superscripts (small letter a-e, h-l, v-z within columns, capital letters A-E, H-L, V-Z within rows) doesn't differ significantly at 5 % level

Table 3. Estimated BOD (mg/l) of poultry slaughter waste and food waste leachate

BOD (mg/l)			
Substrate	**Phase**	**BW-I**	**BW-II**
Summer	P-I	36733.33 ± 430.63^{bB}	2033.33 ± 430.63^{aA}
	P-II	23300.00 ± 999.50^{aB}	2800.00 ± 999.50^{aA}
Monsoon	P-I	22433.33 ± 835.95^{hl}	1850 ± 835.95^{hH}
	P-II	34700 ± 606.81^{il}	2566.67 ± 606.81^{hH}
Post-monsoon	P-I	25466.67 ± 636.77^{vW}	2050.00 ± 636.77^{vV}
	P-II	36350.00 ± 300.42^{wW}	2800.00 ± 300.42^{vV}

Means having same superscripts (small letter a-e, h-l, v-z within columns, capital letters A-E, L, V-Z within rows) doesn't differ significantly at 5 % level

Table 4. Estimated COD (mg/l) of poultry slaughter waste and food waste

COD (mg/l)			
Substrate	Phases	BW-I	BW-II
Season1	P-I	$41975.000 \pm 585.50^{aB}$	3400.000 ± 585.50^{aA}
	P-II	$52575.000 \pm 1076.86^{bB}$	$4283.333 \pm 1076.86^{aA}$
Season 2	P-I	40000 ± 358.17^{hl}	3258.33 ± 358.17^{hH}
	P-II	48933.33 ± 505.66^{il}	4116.67 ± 505.66^{hH}
Season 3	P-I	$41716.667 \pm 625.72^{vW}$	3433.333 ± 625.72^{vV}
	P-II	$52250.000 \pm 790.45^{wW}$	4316.667 ± 790.45^{vV}

Means having same superscripts (small letter a-e, h-l, v-z within columns, capital letters A-E, H-L, V-Z within rows) doesn't differ significantly at 5 % level

Biochemical Oxygen Demand (BOD)

The BOD of the leachate collected during the experimental period is given in Table 3. In BW-I leachate, the highest BOD was found to be in P-I (36733.33 ± 430.63 mg/l) of S-I. The BOD of poultry slaughter waste water reported by Al-Yaqout (2005), Chávez *et al.* (2005) and Wu and Mittal (2012) ranged between 1320 - 5500 mg/l which was very less than that observed for BW-I in the present study, probably because of the dilution with water in poultry slaughter waste water. Wu and Mittal (2012) reported that beef slaughter waste water had a BOD of 14545 mg/l which could be due to the increased content of blood and manure.

The highest BOD for BW-II leachate was during P-II, (2800.000 ± 999.50 mg/l) of S-I. Kylefors (1997) had reported that the BOD of landfill leachate ranged from 49 to 24000 mg/l. Lee *et al.* (2010), reported that young landfill leachate had a BOD of 6350. The present findings were within the range reported by Kylefors (1997) but lower than that reported for young landfill leachate by Lee *et al.* (2010). The difference could be due to the variation in the organic content.

Chemical Oxygen Demand (COD)

The COD of the leachate collected during the experimental period is given in Table

4. The highest COD was found in BW-I leachate was during P-II, (52575.000 ± 1076.86mg/l). The COD observed was similar to that reported by Yuan *et al.* (2012), 40000 to 65000 mg/l for carcass leachate but higher than that reported by Rajakumar *et al.* (2011), 3000-4800 mg/l and Chavez *et al.* (2005), 5800-11600 mg/l for poultry slaughter waste water. This could be due to the fact that the leachate from the slaughter waste had higher level of organic constituents when compared to waste water.

The BW-II leachate showed the highest COD during P-II (4316.67 ± 790.45 mg/l) of S3. Kylefors, (1997) reported that COD of the landfill leachate ranged between 668 and 35000. According to Zamri *et al.* (2016), the COD for stabilised MSW leachate was 3127mg/l. Wang *et al.* (2013) reported a COD of 6200 for raw landfill leachate. In the current study, the COD of BW-I was more than that reported by Zamri *et al.* (2016) but less than that mentioned by Wang *et al.* (2013); values were however within the range reported by Kylefors (1997). In studies by Cruz (2020), the high COD of leachate was attributed to the accumulation of carboxylic acid intermediates. In the current study due to the presence of larva in substrate, part of the intermediates could have been utilised which could be the reason for the lower COD values.

Conclusion

The present study revealed that significant quantities of leachate were produced during the larval bioconversion of food and poultry slaughter wastes. The chemical properties of the leachate revealed that depending on the source of the leachate and age, it could be either acidic or basic and thus could significantly affect the soil chemistry. The high BOD and COD values of the leachate indicate that they need to be pretreated before being released into the environment. The results of this study could form the basis for utilizing the leachate as soil amendment or for use in biomethanation and energy production.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Resource use efficiency of milk production among different types of dairy farms in Kerala#

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Abstract

The present study analysed the resource use efficiency of milk production among different types of dairy farms in Kerala. The area of study and the respondents were selected using a stratified multi-stage random sampling technique. The farmers/farm households were categorized into small farms (1-2 cows), medium (3-10 cows), and large farms (more than ten cows). The relationship between inputs and milk production has been explored through the production function approach. Resource use efficiency was essentially a comparison between the Marginal Value Product (MVP) of an input with its price that gave direction on the use of that particular input in order to maximize profit. The results showed that in small farms, the MVPs of two inputs viz. roughages and labour was significantly less than unity, signifying overutilisation of these inputs. The MVP value of concentrate was significantly higher than unity, indicating their underutilisation in the milk production process. The MVP of concentrate was positive and significantly greater than unity in medium and large farms, also indicating their underutilisation.

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Dairying provides employment as well as stable income to rural people. The dairy farmers were decisive and determined with regard to the actions that were concerned with efficient management of dairy enterprise to obtain better productivity and higher profit (Vidya *et al*., 2009). Maximisation of returns from dairy enterprises requires optimum utilisation of resources required for producing the milk. The profitability of dairy farms is dependent on various aspects such as the productivity of the cows, quality and quantity of the feed materials provided etc. Therefore, the knowledge of resource use efficiency is of great importance for improving the productivity of animals. For achieving optimum resource utilisation of the resources by the dairy farmers, it is necessary to assess the present level of resource use efficiency. It is especially imperative to study the resource use efficiency among different herd-size categories across members of milk cooperative societies. Hence the present study was conducted with a specific objective to find resource use efficiency in milk production among different types of dairy farms in Kerala

Materials and methods

The respondents selected for the present study were dairy farmers, who were members of dairy co-operatives and were enrolled in the Direct Benefit Transfer (DBT) scheme of the Government of Kerala. Since the total population of milk producers who were DBT members was nearly two lakhs, a total sample size of 350 farmers was selected for the study. The farmers/farm households were categorised into small farms (1-3 cows), medium farms (4-10 cows), and large farms (more than 10 cows) (KAU, 2010). Out of the 350 farmers selected for the study, the numbers of small, medium and large farms were fixed as 175, 100, and 75 respectively. A stratified multistage random sampling procedure was used to select the area of study and respondents. In the first stage, the state of Kerala was stratified into five agro-climatic zones (NARP, 1989). In the second stage, one district from each zone (strata) was randomly selected. In the third stage, from each district two blocks were randomly selected. The sample size for each category of farms in each block was determined in proportion to the number of farmers belonging to each category (probability proportion to size technique). For this, all the farmers in the selected blocks were enumerated and classified into small, medium, and large farms based on number of cows. The respondents in each group were chosen randomly in each block, proportional to their number in each block. Primary data were collected by means of observation, in-depth interview and questionnaires.

Milk production function

Production function approach was used to study the effect of different factors on milk production. The factors influencing milk production depends on the character of the milking animal, quality and quantity of feeds fed, labour, management etc. and several other intangible magnitudes that cannot be quantified (e.g. climatic factors, place etc.).

The specification of milk production is as follows

$Y = f(X_1, X_2, X_3, X_4, X_{5.....}X_n)$

Where Y= Value of milk produced per animal per day in Rs.

X_1 = Expenditure on concentrate fed per animal per day in Rs.

X_2 = Expenditure on dry fodder fed per animal per day in Rs.

X_3 = Expenditure on green fodder fed per animal per day in Rs.

X_4= Expenditure on labour (paid+ family) employed per animal per day in Rs.

X_5 = adoption index in percentage

Conceptually any input – output relationship could be expressed as follows

$Y_i = f(X_1, X_2, X_3, \ldots\ldots X_n)$

Where Y_i was the dependent variable or regressed and X1's were the explanatory variables or referred to as regressors. In the present study double log (Cobb-Douglas) production function (Cobb and Douglas, 1928) was fitted whose mathematical form was given below.

Cobb Douglas = $Y=a \Pi^{n}_{i=1} x_{i}^{bi}$

Where Y was the dependent variable, 'a' was the intercept, X_i's were the exogenous variables and bi's are the partial regression coefficient of the respective X_i's.

Marginal value product

Since inputs and outputs were taken in monetary terms, MVPs of all the factors in Cobb-Douglas production function were calculated at respective geometric mean level which was as follows.

$$MVPXi = b_i \frac{\overline{Y}}{\overline{Xi}}$$

Where $\overline{Y}$ = Geometric mean of Y

$\overline{Xi}$ = Geometric mean if ith input

b_i = partial regression co efficient of X_is

The MVPs were compared with the acquisition cost or unit price of the corresponding resources. Use of the concerned resource was recommended to increase if $MVP_{xi} - P_{xi} > 0$ while its use was advised to decrease if $MVP_{xi} - P_{xi} < 0$ the significance of the difference between MVP and unit price of the resource was tested using 't' test which was computed as follows,

$$\text{Calculated } t = \frac{MVPxi - Pxi}{SE\ (MVPxi)}$$

$$SE\ (MVPxi) = \frac{SE\ (bi)\ \overline{Y}}{\overline{Xi}}$$

where SE = Standard Error.

If the difference between MVP and unit price was statistically not significant it indicated optimal use of that particular resource.

Results and discussion

The Cobb-Douglas production function was estimated for different types of farms and overall. The estimated determinants of milk production and coefficient of multiple determinations (R2) of the function is presented in Table 1. The estimated milk production function for different farms revealed that coefficients of concentrate, total roughage and adoption index were positive and statistically significant ($P < 0.01$) in small farms with R^2 as 63.0 per cent, indicating the importance of these inputs in increasing milk production. The labour cost was positive and significant ($P < 0.05$) in small farms. In medium farms, the coefficient of concentrate was highly significant, and roughage was significant at a 5% level. In large farms, the coefficient of concentrate was highly significant. The overall results showed positive and highly significant effects of concentrate and adoption index and significant effect of total roughage ($P < 0.05$) with an R^2 value of 67.0 per cent. This finding assumes more significance as the price of concentrate feed was perceived by dairy farmers as the major constraint in the dairy production (George *et al*., 2017).

Resource use efficiency of milk production

The marginal value product (MVP) of inputs in milk production for different farms are presented in Table 2. The results showed that the MVPs of two inputs, viz. roughages and labour, were significantly less than unity in small farms, signifying overutilisation of these inputs. On the other hand, the MVP value of concentrate was significantly higher than unity, indicating their underutilisation in the milk production process. In medium farms, the MVP of concentrate was positive and significantly greater than unity, indicating their underutilisation. In large farms, the MVP value of concentrate was positive and significantly greater than unity, indicating their sunderutilisation.

Similar works on resource use efficiency of milk were conducted in different parts of the country showing both supporting and contrary results. Kumar and Singh (2004) conducted a study in Tamil Nadu and found that in crossbred cows, the spending on feed factors like concentrate and dry roughage significantly affect the returns from the enterprise, whereas expenditure on green fodder was non-significant. They also concluded that MVPs of dry fodder and concentrates were significantly more than unity, indicating their underutilisation, while green fodder and labour were non significantly different from unity, indicating their optimal use. Mahajan and Chauhan (2011), Bardhan and Sharma (2013), Sharma *et al*. (2014), Rangnath *et al*. (2015) and Prusty and Tripathy (2016) also made investigations on resource use efficiency with similar results.

Table 1. Estimated Coefficients of milk production function

Farm size/ (Observations)	Parameter	Regression coefficient		R2 (%)
		B	Std. Error	
Small (175)	(Constant)	-0.958	0.922	63
	Concentrate	0.619**	0.051	
	Roughage	0.191**	0.045	
	Veterinary Service	-0.008	0.029	
	Labour cost	0.088*	0.04	
	Adoption Index	0.589**	0.218	
Medium (100)	(Constant)	0.153	1.229	81.4
	Concentrate	0.734**	0.058	
	Roughage	0.091*	0.046	
	Veterinary Service	-0.045	0.045	
	Labour cost	0.018	0.08	
	Adoption Index	0.377	0.31	
Large (75)	(Constant)	4.224	0.94	65.7
	Concentrate	0.585**	0.056	
	Roughage	0.018	0.024	
	Veterinary Service	-0.007	0.061	
	Labour cost	-0.039	0.064	
	Adoption Index	-0.262	0.227	
Overall (350)	(Constant)	-0.126	0.599	67.0
	Concentrate	0.671**	0.032	
	Roughage	0.053*	0.022	
	Veterinary Service	0.002	0.023	
	Labour cost	0.031	0.032	
	Adoption Index	0.512**	0.141	

** Significant at 1% level

* Significant at 5% level

Table 2. Marginal value products of various inputs under different farm sizes

Farm size	Inputs	MVP
Small	Conc.	1.54**(0.13)
	Roughages	0.59** (0.14)
	Labour	0.21** (0.1)
Medium	Conc.	2.02** (0.16)
	Roughages	0.63(0.32)
Large	Conc.	1.68**(0.16)

Figures in the parenthesis are standard errors of regression coefficients.
** Significant at 1% level

Conclusion

The analysis of resource use efficiency of milk production using the Cobb-Douglas production function demonstrated the importance of inputs like concentrate, total roughage, and labour and adoption index in increasing milk production in small farms. The coefficient of concentrate was significant in medium and large farms also. In small farms, the MVPs of two inputs, viz. roughages and labour, were less than unity, signifying over utilisation of these inputs. On the other hand, the MVP value of concentrate was significantly higher than unity, indicating their underutilisation in the milk production process. If an input is over-utilized, the quantity of that input can be reduced without affecting the output of milk, and if an input is underutilised, its amount can be increased to improve the production of milk. As per the results of the present study, the quantity of concentrate can be increased for improving milk production in all types of farms.

Conflict of interest

The authors declare that they have no conflict of interest

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Effect of bedding systems and thermal analysis on milk yield of crossbred dairy cows

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Abstract

The current research was carried out to assess the effect of various bedding material on thermal image analysis and yield of milk in dairy cows. Twenty four crossbred cows at the Cattle farm of the Instructional Livestock Farm Complex, Pookode, Wayanad District in Kerala state formed the group in the present research for one lactation period including three seasons viz., summer (February to May), monsoon (June to September) and post monsoon (October to January). T_1 was the control group in which the cows were maintained on concrete floor with no bedding material. Rubber mats (T_2) and coir pith (T_3) were provided on concrete floor. In T_4 Dried solid manure (DSM) on concrete floor was provided at the rate of 7.5 cm thickness as bedding. Thermal image analysis of the heat generated from the animal body, surface of the floor and roof was recorded. The cows maintained on concrete floors (37.79 ± 0.21 °C) and rubber mats (37.56 ± 0.28 °C) generated significantly higher (P<0.05) amounts of heat followed by those kept on DSM (28.49 ± 0.28 °C) and coir pith (25.46 ± 0.26°C). The floor and roof temperatures were higher during summer and post monsoon and lower in the monsoon season. Daily milk yields from the experimental animals were recorded and analysed. The cows maintained on concrete floor had the lowest overall daily milk yield (8.95 ± 0.22) while the cows on coir pith bedding had the highest yield (9.98 ± 0.30). The cows on DSM and rubber mats had an overall mean milk yield of 9.48 ± 0.22 and 9.26 ± 0.20 kg, respectively. Coir pith and dried solid manure as bedding material could be recommended for use by dairy farmers when compared to rubber mats and concrete floor bedding for improving cow comfort and milk production.

Keywords: *Crossbred cows, bedding systems, thermal imaging, milk yield*

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As per 20th livestock census in 2019, 192.49 million numbers of cattle (ranking second in the world population) are available in India and 93 percent of the cattle population are crossbreds in Kerala. Even though crossbred cows are more vulnerable to disease and climatic stress, they are docile and better producers of milk. Hence good care and welfare and comfortable housing with soft beddings are required. Cow comfort under an intensive management system is economically important as it significantly affects the feed intake of the animal, as well as production and reproduction. The primary consequences of reduction in comfort in dairy cows leads to loss of energy, accompanying stress, consequent reduction in feed intake and milk yield (Praveenkumar *et al*.,2022). The World Organisation for Animal Health (OIE, 2008) has propounded five freedoms in relation to welfare of animals and among these, one is the freedom from physical and thermal discomfort by providing access to shelter and a comfortable resting area. Other mentions in the aforementioned document on this line include the freedom to express normal behavioural patterns by providing sufficient space, proper facilities and the company of other animals of the same kind. Availability, cost, design, comfort, ease of use and reuse are the important factors influencing the selection of suitable bedding material on dairy farms. The most commonly used bedding material in cow housing systems are sawdust, wood shavings and sand (Oliveira *et al*., 2019). Other material, including straw, peanut shells and woodchips (Leso *et al.,* 2020) are commonly used bedding material. The demand for traditional bedding material has driven up prices, pushing farmers to look for alternative bedding material. A complete understanding of the nature of alternative material and their characteristics with regard to their use and handling as bedding material is crucial (Agnew and Leonard, 2013) while making a choice of the same.

During the extraction of coir fibre from coconut husk, a ligno-cellulosic biomass, coir pith is formed. It is a comfortable, suitable and animal friendly bedding material. It is ideal for use as bedding for cows due to innate moisture absorbing quality and soft bed cushioning effects. Cattle housing should be well ventilated regardless of the bedding material used, but particularly so when recycled manure solids are used and along with ventilation, adequate drainage should also be installed to ensure a drier environment that would discourage pathogen growth (Leach *et al.,* 2015). Interest in using recycled manure solids (RMS) as a bedding material for dairy cows has grown among commercial milk producers for most farms. The cost of milk production in the state of Kerala was very high, and the milk price has to be adjusted accordingly so that dairy farming becomes a lucrative livelihood opportunity for poor farmers (Sabin *et al*., 2022). Extensive research has amply proved that the use of bedding materials for dairy cattle improves animal comfort and increases milk production. However, research on the use of various types of bedding material for crossbred dairy cattle is scanty. Since the bedding material has a direct relationship with the welfare of the cows, the present study was undertaken to evaluate the thermal image analysis and milk yield in different bedding systems in crossbred cows.

Materials and methods

The study was carried out at the Cattle farm of the Instructional Livestock Farm Complex, Pookode, Wayanad District in Kerala state, located at 11° 32' 18.5 (North) longitude and 76° 01' 14.15 (East) latitude, at an altitude of 867 m above the mean sea level. The locale of study was endowed with humid climate with maximum rainfall by South West monsoon from June to September and North East monsoon from October to November. The study was carried out for one lactation period of 305 days spread over three different seasons as described by Biya (2011) *viz.,* summer months (Feb-May) (25-35°C), monsoon months (June-Sep) (24-31°C) and post monsoon months (Oct-Jan) (20-30°C). Twenty four crossbred dairy cows in early stage of lactation, between four to six years of age were selected for the study. The animals were divided into four groups with six animals in each group with regard to their average body weight of 270-300 kg in 2nd to 4th parity with milk yield of 8.15 to 11.50 kg as uniformly as possible before the start of experimentation.

The cows were maintained under four bedding systems. The animals were let loose in the shed except during feeding and milking time. Floor space of 13 sq. m and manger space of 1.2 m length and 0.6 m width were provided per cow. Dung was removed manually in the mornings and evenings. Animals were washed outside the shed during the trial period. Animals were fed as per ICAR (2013) standards. Daily concentrate ration was fed at 5.00 AM and 2.00 PM and roughage at 10.00 AM and 3.00 PM. Water was provided *ad libitum*. All the treatment groups including control were housed in East-West oriented sheds in a face-to-face arrangement.

Six experimental animals were maintained in the existing management system, *viz.,* concrete floor without any bedding material (T_1). This group was considered as the control group. Rubber mats on concrete floor of 1.2m × 1.8m × 0.025m area were used for six experimental animals (T_2). All other activities including the feeding regime were followed as per routine practice. The rubber mat used in experiment was 16 mm thick, 6'× 4' in size and weighed 40 kg. Coir pith was provided at the rate of 7.5 cm thickness as bedding (T_3). Dried solid manure was provided at the rate of 7.5 cm thickness as bedding (T_4). The moisture content of the DSM was maintained below 25

Table 1. Mean heat generated from cow body in different bedding systems during different seasons

Treatments (n=6)		At 08.00 AM (Mean ± SE) (°C)				At 01.00 PM (Mean ± SE) (°C)				At 05.00 PM (Mean ± SE) (°C)			
		S	M	PM	S	M	PM	S	M	PM	S	M	PM
T_1	Concrete	38.00 ± 0.33	36.46 ± 0.20	37.67 ± 0.47	37.14 ± 0.21^{a}	37.58 ± 0.33	37.35 ± 0.25	36.06 ± 0.49	37.39± 0.21^{a}	37.93 ± 0.29	37.18 ± 0.19	37.24 ± 0.41	37.77 ± 0.20^{a}
T_2	Rubber mat	38.35 ± 0.46	36.37 ± 0.21	37.86± 0.47	37.36 ± 0.26^{a}	38.39 ± 0.54	37.41± 0.20	37.64 ± 0.39	37.56 ± 0.28^{a}	38.68 ± 0.56	37.12 ± 0.38	37.27 ± 0.36	37.89 ± 0.29^{a}
T_3	Coir pith	25.43 ± 0.63	24.25 ± 0.54	24.72 ± 0.34	25.13 ± 0.29^{c}	26.02 ± 0.49	24.48 ± 0.47	25.87 ± 0.27	25.46± 0.26^{c}	26.15± 0.45	24.84 ± 0.57	25.95 ± 0.27	25.23 ± 0.27^{c}
T_4	DSM	28.03± 0.43	27.06 ± 0.23	27.79 ± 0.52	27.65 ± 0.23^{b}	28.74 ± 0.54	27.39 ± 0.26	28.04 ± 0.64	28.49± 0.28^{b}	28.57 ± 0.51	27.35 ± 0.24	28.16 ± 0.50	28.11 ± 0.24^{b}
Overall (Mean± SE)		32.76± 1.17^{A}	31.02 ± 1.14^{C}	32.13 ± 1.13^{B}	31.39 ± 0.65	31.89 ± 1.16^{A}	31.08 ± 1.15^{C}	31.37 ± 1.14^{B}	32.13± 0.66	32.72 ± 1.2^{A}	31.32 ± 1.20^{C}	31.56 ± 1.16^{B}	31.45 ± 0.68

Means with different superscripts (a-c in rows, A-B in columns) differ significantly (P<0.05)

Table 2. Mean daily heat generated from floor and roof during different seasons

Season	Floor surface temp (°C)			Roof surface temp (°C)		
	At 8 AM	At 1 PM	At 5 PM	At 8 AM	At 1 PM	At 5 PM
Summer	26.17 ± 0.37^{a}	27.53 ± 0.35^{a}	26.26 ± 0.34^{a}	41.03 ± 0.47^{a}	42.78 ± 0.53^{a}	41.83 ± 0.33^{a}
Monsoon	23.96 ± 0.37^{c}	24.49 ± 0.40^{c}	24.20 ± 0.37^{c}	37.34 ± 0.40^{c}	37.67 ± 0.41^{c}	37.39 ± 0.41^{c}
Post Monsoon	25.36 ± 0.28^{b}	26.20 ± 0.22^{b}	25.68 ± 0.22^{b}	40.40 ± 0.10^{b}	40.56 ± 0.10^{b}	40.29 ± 0.12^{b}

Means with different superscripts (a-c in rows) differ significantly (P<0.01)

Table 3. Mean daily milk yield in different bedding systems during different seasons

Treatments (n=6)		Daily milk yield (Mean± SE) (kg)			
		Summer	Monsoon	Post monsoon	Overall
T_1	Concrete	8.20 ± 0.07	9.31 ± 0.03	9.28 ± 0.03	8.95 ± 0.22^{d}
T_2	Rubber mat	8.23 ± 0.01	10.28 ± 0.03	9.30 ± 0.03	9.26 ± 0.20^{c}
T_3	Coir pith	8.35 ± 0.04	11.28 ± 0.04	10.35 ± 0.03	9.98 ± 0.30^{a}
T_4	DSM	8.28 ± 0.01	10.75 ± 0.03	9.41 ± 0.05	9.48 ± 0.22^{b}
(Mean± SE)		8.26 ± 0.03^{C}	10.66 ± 0.09^{A}	9.52 ± 0.15^{B}	9.34 ± 0.13

Means with different superscripts (a-d in rows, A-C in columns) differ significantly (P<0.05)

per cent above which the wet material was replaced by dried bedding (Li *et al.,* 2008). Concentrate and green fodder was fed as per the routine schedule. All other activities including the feeding regime were followed as per standard practice.

The coir pith and DSM were sundried and treated with two per cent calcium hydroxide to destroy pathogens and lime was mixed in homogeneously (Gerba and Smith, 2005) before each season of the experiment. Thermal image analysis of the heat generated from animal body, surface of floor and roof was recorded by using an FLIR® infrared camera three times in a day at 8.00 AM, 1.00 PM and 5.00 PM at weekly intervals. Data on daily milk yield (kg) were recorded with respect to all experimental animals both in the morning at 5.30 AM and in the afternoon at 2.30 PM. Two-way ANOVA with interaction effect was performed to study the effect of different bedding material and seasons on milk yield of cows.

Results and discussion

Heat generated from animal body

The overall mean heat generated by the cows managed under different bedding material was recorded three times in a day at, at 8.00 AM, 1.00 PM and 5.00 PM, at weekly intervals. In order to understand the variations in the generation of heat at different time intervals, three separate analyses were performed with the values recorded at three different time intervals as mentioned above and the mean values are furnished in Table 1. The results of two-way ANOVA revealed that the overall mean heat generation from the animals maintained in different bedding material differed with the seasonal variations as the interaction effect was significant (P<0.05). Also, the type of bedding material had significant effect on the amount of heat generated at 1:00 PM. The cows maintained on concrete floor (37.79 ± 0.21) and rubber mat (37.56 ± 0.28) generated significantly higher (P<0.05) amounts of heat followed by DSM (28.49 ± 0.28) and coir pith (25.46 ± 0.26°C).

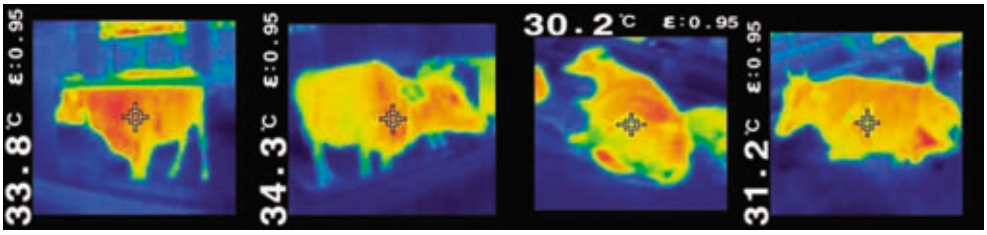
The overall mean heat generated from the body of the cow reared on different bedding systems were in decreasing order from rubber mats, concrete floor, DSM and coir pith at 8:00 AM and 5:00 PM. In general, it was noted that the animals maintained on coir pith and DSM generated comparatively lower body heat while the cows maintained on concrete and rubber mat had higher body heat generation irrespective of the seasons and timing of recording. However, the overall body heat generation at 1.00 PM was significantly higher (P<0.05) than at 8.00 AM and 5.00 PM during summer. The same pattern was found during the monsoon and post monsoon seasons. Thermal images of cow body heat in different seasons, *viz.,* summer, monsoon and post monsoon are depicted in Fig. 1.

This study reiterates the report of Kunc *et al*. (2007) where they mentioned that IRT could be used as a potential non-invasive and non-contact heat-detecting technology and the infrared camera measured the infrared radiation emitted from an object.

Heat generated from floor and roof

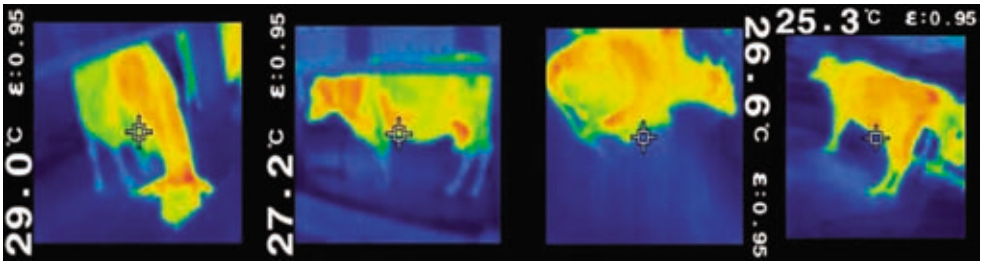
The mean daily heat generated from floor and roof is presented in Table 2. The floor temperature was higher (27.53 ± 0.35) in summer followed by post monsoon (26.20 ± 0.22) and lower in monsoon (24.49 ± 0.40°C) at 1.00 PM with significant difference (P<0.01). Ae similar trend was noted for floor temperature at 8:00 AM and 5:00 PM during the three different seasons. The roof temperature was also higher (42.78 ± 0.53) in summer followed by post monsoon (40.56 ± 0.10) and monsoon (37.67 ± 0.41°C) at 1.00 PM with significant difference (P<0.01). The same pattern was observed in during monsoon and post monsoon at 8:00 AM and 5:00 PM. In general, both the floor and roof showed higher temperature during summer and lower temperature during post monsoon and monsoon.

Knizkova *et al*. (2007) reported temperatures of 27.95 ± 0.15 on the floor and 37.17 ± 0.32°C on the roof of cattle sheds during the summer season than other seasons which concurs with the findings of the present study. They also noted that the effect of weather conditions, circadian and ultradian rhythms, time of feeding, milking, lying and rumination



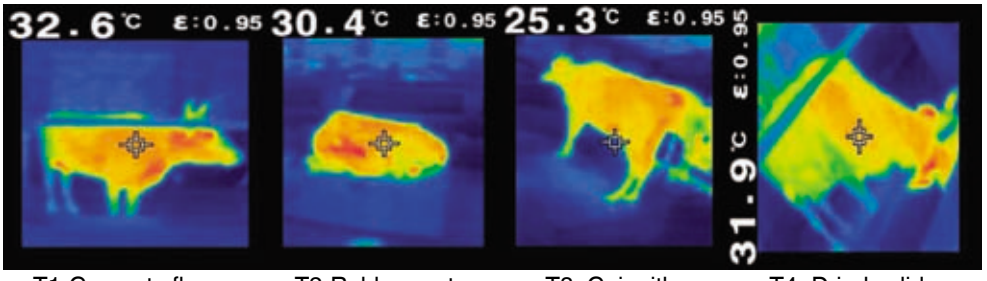
T1-Concrete floor T2-Rubber mats T3 -Coir pith T4 -Dried solid manure

a. Summer



T1-Concrete floor T2-Rubber mats T3 -Coir pith T4 -Dried solid manure

b. Monsoon



T1-Concrete floor T2-Rubber mats T3 -Coir pith T4 -Dried solid manure

c. Post monsoon

Fig. 1. Thermal images of cow body heat in different seasons

etc., needed to be considered along with factors like sunlight, moisture, dirt, weather condition, *etc*., during the measurement of temperature using IRT.

Milk yield

The mean daily milk yield of cows on different bedding material is presented in Table 3. The results revealed that the type of bedding material, season and the interaction between seasons and bedding material significantly altered the mean milk yield of cows (P<0.05). The F-value for the interaction (76.12) and between groups (672.40) and seasons (4066.99) were found to be statistically significant.

The cows maintained on concrete floor had the lowest overall daily milk yield (8.66 ± 0.22) while the cows on coir pith had the highest yield (9.98 ± 0.30). The cows on DSM and rubber mats had the overall mean milk yield of 9.48 ± 0.22 and 9.26 ± 0.20 kg, respectively. The per cent increase in milk yield was 12.14 in cows maintained on concrete floor, which served as the control group followed by 15.33 on rubber mat, 17.21 on DSM and 19.50 on coir pith bedding. Before the start of trial, the overall mean milk yield was 8.24 ± 0.05, 8.25 ± 0.04, 8.27 ± 0.02 and 8.26 ± 0.06 for cows reared on concrete floor, rubber mats, coir pith and DSM bedding systems.

The results of Kremer *et al*. (2007) as they reported greater activity and better overall milk yield of high-yielding dairy cows which were on elastic rubber mats (9.28 ± 0.12) than that on concrete floor (8.68 ± 0.12 kg) in a loose housing system was complementary to the present study. The findings of Singla *et*

al. (2007) are in agreement with this study, as they reported mean milk yield of 11.27, 10.56, 9.82 and 9.55 L/animal/d in herds provided with paddy straw bedding material and 11.34, 10.32, 9.31 and 9.26 L/animal/d in coir pith bedding in depth of 30 cm, 20 cm, 10 cm on concrete floor and in the present study bedding material of 7.5 cm thickness was provided.

From Table 3. it may also be noted that the mean milk yield of cows maintained on different bedding materials was also influenced by the seasonal variations as the differences of overall means of milk yield during different seasons within the cow groups were statistically significant (P<0.05). Moreover, the mean values for different seasons ranged from 8.26 ± 0.03 kg in summer to 10.66 ± 0.09kg in monsoon. The results are in agreement to Barberg *et al*. (2007) as they noted increase in milk production of 9.57 ±0.12 kg in compost bedded pack than bedded with dry fine wood shavings or sawdust (9.76 ± 0.03 kg). Singh *et al*. (2015) obtained the average highest seasonal milk production of 10.52 ± 0.12 and 9.54 ± 0.14 kg in crossbred during winter and summer season, respectively with highly significant difference (P<0.05) in seasonal variation and milk production performance which coincides with the present study.

Conclusion

In the present study, the cows maintained on concrete floor (37.79 ± 0.21) and rubber mat (37.56 ± 0.28) generated significantly higher (P<0.05) amounts of heat followed by DSM (28.49 ± 0.28) and coir pith (25.46 ± 0.26°C). The cows maintained on concrete floor had the lowest overall daily milk yield (8.95 ± 0.22) while the cows on coir pith had the highest yield (9.98 ± 0.30 kg). Thus, coir pith and dried solid manure as bedding material could be recommended to dairy farmers when compared to rubber mats and concrete floor for improving cow comfort and milk production.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Antibiofilm activity of berberine and capsaicin in combination with quinolones against S*taphylococcus aureus* from bovine mastitis#

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Abstract

Antimicrobial resistance which causes failure of antibiotic therapy has become a serious global issue nowadays. Bacteria develop resistance towards an antibacterial agent via many mechanisms. Biofilm formation by bacteria poses severe threat to the treatment of infections. The combination of plant molecules with antibiotics can combat antimicrobial resistance. In the present study, antibiofilm activity of two plant molecules berberine and capsaicin was investigated against Staphylococcus aureus isolates from bovine mastitis cases. Antibiotic sensitivity testing revealed that berberine and capsaicin when combined with quinolones increased the diameter of zone of inhibition in a dose dependent manner. In vitro biofilm assay using congo red agar plate method revealed the antibiofilm activity of berberine and capsaicin in combination with quinolone antibiotics. The study concludes that the combination of phytoconstituents with antibiotics can alleviate resistance mechanisms in bacteria.

Keywords: *Antimicrobial resistance, biofilm, Staphylococcus aureus, bovine mastitis*

Antimicrobial resistance (AMR) has emerged as one of the most important public health risks, posing substantial challenges to the prevention and treatment of chronic diseases. Despite several steps taken in recent decades to address this issue, the worldwide AMR graph indicates no evidence of slowing down. The misuse and overuse of various antibacterial agents

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in the health care, as well as in the agricultural industry, are thought to be the primary causes of antimicrobial resistance (Dadgostar, 2019). Drug inactivation/alteration, modification of drug binding sites/targets, changes in cell permeability resulting in lower intracellular drug accumulation and biofilm formation are all different mechanisms of drug resistance in bacteria (Santajit and Indrawattana, 2016). In livestock sector, mastitis poses a serious threat as the development of resistance among mastitis causing pathogens hold back the treatment (Jisha *et al*., 2020). In dairy cattle, *Staphylococcus aureus* is a common cause of intra-mammary infection. Its ability to endure inside the udder is based on the presence of important mechanisms such as the ability to form biofilm, polysaccharide capsules, small colony variants and the ability to invade cells, all of which will protect *S. aureus* from the cow's innate and adaptive immune response, as well as antibiotics that are no longer considered adequate against the organism (Zaatout *et al*., 2020; Jose *et al*., 2021).

Biofilm is an assemblage of microbial cells that has been irrevocably attached to a surface and is encased in a polysaccharide matrix. Biofilms can grow on a number of surfaces, including living tissues, medical devices, piping in industrial or potable water systems and natural aquatic systems. Biofilm-associated organisms differ from their planktonic (freely suspended) counterparts with respect to the genes that are transcribed (Donlan, 2002). When compared to their planktonic counterparts, bacterial cells in biofilms are 10 to 1,000 times less susceptible to specific antimicrobial agents. Poor antibiotic penetration into the polysaccharide matrix, the arbitrary presence of cells with a resistant phenotype and the presence of either non-growing cells or cells that induced stress responses under undesirable chemical conditions within the biofilm matrix, all contribute to the reduced susceptibility of antimicrobials against biofilm producing bacteria (Balcazar *et al*., 2015).

Bacterial biofilms pose significant public health concern because of their ability to withstand antibiotics, immune mechanisms and other external stimuli contributing to persisting chronic infections. As a result, the biofilm matrix provide bacteria with additional capability allowing them to not only withstand hard conditions but also become resistant to antibiotics, resulting in the development of multidrug resistant, extensively drug resistant and totally drug resistant strains (Sharma *et al*., 2019). The majority of antimicrobial therapies have been developed and tested on bacteria that live in a planktonic (free-living) state. As a result, antimicrobial therapies are frequently ineffective against pathogenic biofilms, which could be about 1000 times more resistant to antimicrobial treatments. Biofilm recalcitrance makes it incredibly difficult to successfully treat and remove them (Verderosa *et al*., 2019). As a result, novel solutions for bacterial biofilm prevention, dispersal and treatment are the need of the hour.

The present study was undertaken with the aim to evaluate the antibiofilm activity of two plant molecules berberine, a benzylisoquinoline alkaloid from *Berberis vulgaris* L. and capsaicin, natural proto-alkaloid found in hot peppers (*Capsicum annuum* L.) in combination with quinolones against *S. aureus* isolates from bovine mastitis.

Materials and methods

Collection of S. aureus isolates

The *S. aureus* isolates from bovine mastitis milk samples were procured from Department of Veterinary Microbiology, Teaching Veterinary Clinical Complex, College of Veterinary and Animal Sciences, Mannuthy, Thrissur.

Antibiotic sensitivity test (ABST)

Antibiotic sensitivity testing was performed by Kirby-Bauer disc diffusion assay as per the guidelines provided by Clinical and Laboratory Standards Institute (CLSI, 2012). Bacterial suspension adjusted to 0.5 McFarland standards were swabbed on sterile Mueller Hinton agar plates. The test compounds berberine and capsaicin (obtained from Sigma Aldrich in pure form) were dissolved in dimethyl sulphoxide (DMSO) and two fold serial dilutions of test compounds ranging from 8 g/L to 0.25

g/L were used in the study. Twenty microlitres of dilutions (8 g/L to 0.25 g/L) of berberine and capsaicin were impregnated into each of the antibiotic discs that already contained nalidixic acid (30 mcg), norfloxacin (10 mcg) and enrofloxacin (10mcg) separately. The antibiotic discs alone and the vehicle control (DMSO) were also added. Discs were placed on the agar plate using sterile forceps. Plates were then incubated immediately at 37°C for 16-18 h. After incubation, the zone of inhibition was measured and interpreted according to CLSI criteria. All tests were done in triplicates against six *S. aureus* isolates.

Biofilm assay

The ability of *S. aureus* isolates to form biofilm was characterised phenotypically by Congo red agar (CRA) plate method as described by Melo *et al*. (2013). The *S. aureus* isolates were cultured in a CRA medium for the assay. The CRA medium comprised of brain heart infusion agar (52 g), saccharose (36 g) and congo red dye (0.8 g) per litre of autoclaved distilled water. The plates were then inoculated with the isolates and incubated for 24 h at 37°C aerobically. A positive result was indicated by black colonies with a dry crystalline consistency while pink colonies with occasional darkening resembling a bull's eye appearance indicated a negative result (Freeman *et al*., 1989).

The ability of the test compounds berberine and capsaicin to inhibit biofilm formation was assessed by the CRA plate method. The antibiofilm activity was assessed in *S. aureus* isolates treated with berberine and capsaicin alone and in combination

Table 1. Zone of inhibition (mm) of quinolone resistant *S. aureus* against enrofloxacin with different combinations of berberine and capsaicin

Treatment	Zone of Inhibition (mm) Mean ± SE	
	EXB	EXC
Enrofloxacin (10 mcg)	11.67 ± 0.21^{f}	11.67 ± 0.21^{g}
Enrofloxacin + test 8g/L	18.83 ± 0.7^{a}	18.67 ± 0.56^{a}
Enrofloxacin + test 4g/L	18 ± 0.58^{ab}	17.67 ± 0.56^{ab}
Enrofloxacin + test 2g/L	17.17 ± 0.48^{abc}	16.83 ± 0.48^{abc}
Enrofloxacin + test 1g/L	16.17 ± 0.48^{bc}	16.17 ± 0.65^{bcd}
Enrofloxacin + test 0.5g/L	14.17 ± 0.4^{cde}	14.17 ± 0.4^{def}
Enrofloxacin + test 0.25g/L	13.67 ± 0.21^{def}	13.83 ± 0.31^{efg}
F value	31.90^{***}	25.54^{***}
P value	0.0001	0.0001

*** Significant at 0.05 level, n=6, r=3, Means bearing varying superscript differ significantly in a column. EXB →enrofloxacin + berberine, EXC→ enrofloxacin + capsaicin

Table 2. Zone of inhibition (mm) of quinolone resistant *S. aureus* against norfloxacin with different combinations of berberine and capsaicin

Treatment	Zone of Inhibition (mm) Mean ± SE	
	NXB	NXC
Norfloxacin (10 mcg)	0^{c}	0^{b}
Norfloxacin + test 8g/L	12.17 ± 0.4^{a}	12.5 ± 0.22^{a}
Norfloxacin + test 4g/L	11 ± 0.37^{a}	11.83 ± 0.17^{a}
Norfloxacin + test 2g/L	10.5 ± 0.22^{a}	11 ± 0.26^{a}
Norfloxacin + test 1g/L	8.17 ± 0.98^{ab}	10.17 ± 0.17^{a}
Norfloxacin + test 0.5g/L	0^{c}	0^{b}
Norfloxacin + test 0.25g/L	0^{c}	0^{b}
F value	33.36^{***}	84.68^{***}
P value	0.0001	0.0001

*** Significant at 0.05 level, n=6, r=3, Means bearing varying superscript differ significantly in a column. NXB →norfloxacin + berberine, NXC→ norfloxacin + capsaicin

Table 3. Zone of inhibition (mm) of quinolone resistant *S. aureus* against nalidixic acid with different combinations of berberine and capsaicin

Treatment	Zone of Inhibition (mm) Mean ± SE	
	NAB	NAC
Nalidixic acid (30 mcg)	0^{c}	0^{d}
Nalidixic acid + test 8g/L	11.5 ± 0.5^{a}	10.5 ± 0.22^{a}
Nalidixic acid + test 4g/L	11 ± 0.45^{a}	8.67 ± 1.74^{ab}
Nalidixic acid + test 2g/L	7 ± 2.24^{ab}	5 ± 2.24^{bc}
Nalidixic acid + test 1g/L	3.67 ± 2.33^{b}	1.67 ± 1.67^{cd}
Nalidixic acid + test 0.5g/L	0^{c}	0^{d}
Nalidixic acid + test 0.25g/L	0^{c}	0^{d}
F value	18.16^{***}	15.81^{***}
P value	0.0001	0.0001

*** Significant at 0.05 level, n=6, r=3, Means bearing varying superscript differ significantly in a column. NAB →nalidixic acid + berberine, NAC→ nalidixic acid+ capsaicin

with quinolone antibiotics enrofloxacin, norfloxacin and nalidixic acid at sub-inhibitory concentrations. One milliliter dilution of test compounds berberine and capsaicin were made separately in sterile test tubes by two fold serial dilution ranging from 8 g/L to 0.25 g/L. Then one milliliter of antibiotics at sub inhibitory concentration level was added to each test tubes followed by the addition of one milliliter of bacterial suspension (adjusted to 0.5McFarland standard). Then, the tubes were incubated at 37ºC for 24 h. After 24 h of incubation, the sterile CRA plates were inoculated with respective isolates. The biofilm-producing *S. aureus* isolates served as the positive control. The plates were incubated at 37ºC for 48 h aerobically. The experiment was done in triplicates. Antibiofilm formation was assessed by analysing the colony morphology. Inhibition of biofilm was indicated by the formation of pink colonies in the CRA plate while biofilm producers remain as dry black crystalline colonies. Indeterminate result was indicated by the darkening of colonies with the absence of dry, crystalline colonial morphology.

Results and discussion

Antibiotic sensitivity test (ABST)

Mastitis is an important disease causing economic burden and production loss in livestock sector. The prevalent multidrug resistant (MDR) isolates in bovine mastitis impedes the treatment strategies thereby resulting in loss of producing animals.

Antibiotic sensitivity test of *S. aureus* isolates against enrofloxacin showed a zone of inhibition of 11.67±0.21mm and against norfloxacin and nalidixic acid no measurable zone of inhibition was observed. When enrofloxacin combined with berberine or capsaicin, there was an increase in zone of inhibition observed with a maximum of 18.83±0.7 mm when combined with berberine and 18.67±0.56 mm when combined with capsaicin (Table 1, Fig.1). The combinations of norfloxacin with berberine at 8g/L showed a zone of inhibition of 12.17±0.4 mm and norfloxacin with capsaicin at 8g/L showed a zone of inhibition measuring 12.5±0.22 mm. (Table 2, Fig.2). When nalidixic acid combined with different combinations of berberine or capsaicin there was an increase in the zone of inhibition at very high concentrations exhibiting 11.5±0.5mm for berberine and 10.5±0.22mm for capsaicin (Table 3, Fig. 3).

The results of the current study is in accordance with the findings of Wojtyczka *et al*. (2014) where, berberine in combination with antibiotics increased the zone of inhibition more than 5mm against coagulase negative Staphylococcal strains. The study of Nisha *et al*. (2020) on the antibacterial activity of capsaicin against tetracycline resistant *E. coli* isolates reported that capsaicin increased the zone of inhibition of tetracycline in a dose dependent manner when treated against tetracycline resistant *E. coli*. The present study showed that berberine and capsaicin when combined along with antibiotics potentiated its activity.

The disc diffusion assay on combinations of berberine and capsaicin with antibiotics is scarcely documented. However, berberine and capsaicin pose weak antibacterial activity which can be explained through the findings of Chu *et al*. (2016) and Marini *et al*. (2015).

Biofilm assay

The pathogenicity of *S. aureus* is linked to its ability to release toxins and other extracellular components, its resistance to phagocytosis as well as its ability to adhere to host surfaces and form biofilm (Vasudevan *et al*., 2003). Biofilms are complex surface-attached congregations of bacteria bound together by self-produced polymer matrixes, which are mostly made of extracellular DNAs, polysaccharides and secreted proteins (Muhammed *et al*., 2020). The persistent infections of bovine mastitis can be linked to formation of biofilm by microbes because adhesive colonies are contained by a vast exopolysaccharide matrix. Apart from evasion of host defence mechanisms, one of the most striking characteristics of biofilm producing pathogenic strains is antibiotic resistance, which complicates the treatment of clinical cases (Shah *et al*., 2019). The structural integrity, physiological and chemical characteristics of biofilm vary between microorganism species and are dependent on the development of extracellular matrix (Silva *et al*., 2020). Thus inhibition of biofilm via conventional antimicrobials is a challenging task especially against MDR bacterial isolates.

Congo red agar (CRA) plate method is a sensitive diagnostic tool to assess the formation of biofilm by bacteria. Congo red dye can aid in visualizing the amyloid fibers and overexpressing exopolysaccharide matrix associated with the biofilm (Jones and Wozniak, 2017).

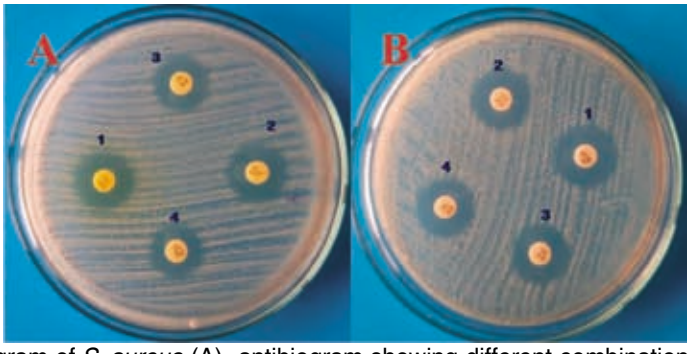

Fig. 1. Antibiogram of *S. aureus* (A)- antibiogram showing different combinations of enrofloxacin with berberine (B)- antibiogram showing different combinations of enrofloxacin with capsaicin. 1→ 8 g/L, 2→ 4 g/L, 3→ 2 g/L, and 4→ 1 g/L

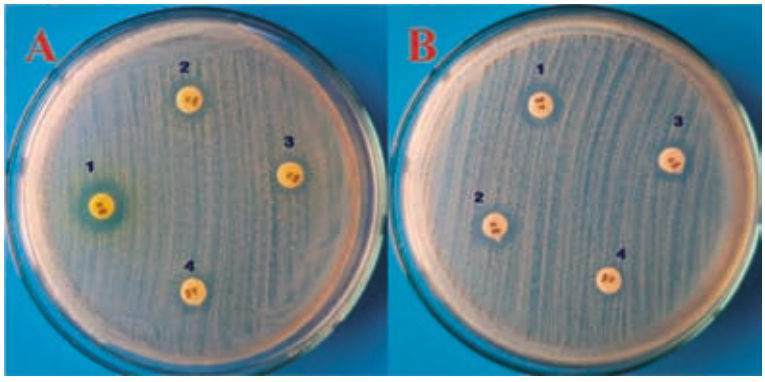

Fig. 2. Antibiogram of *S. aureus* (A)- antibiogram showing different combinations of norfloxacin with berberine (B)- antibiogram showing different combinations of norfloxacin with capsaicin. 1→ 8 g/L, 2→ 4 g/L, 3→ 2 g/L, and 4→ 1 g/L

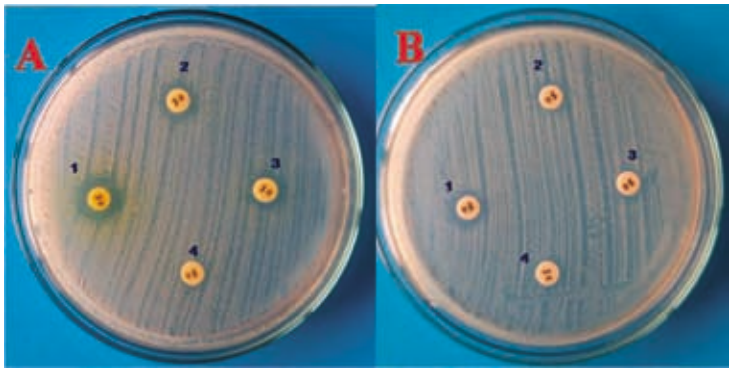

Fig. 3. Antibiogram of *S. aureus* (A)- antibiogram showing different combinations of nalidixic acid with berberine (B)- antibiogram showing different combinations of nalidixic acid with capsaicin. 1→ 8 g/L, 2→ 4 g/L, 3→ 2 g/L, and 4→ 1 g/L

Fig. 4. Biofilm producing *S. aureus*

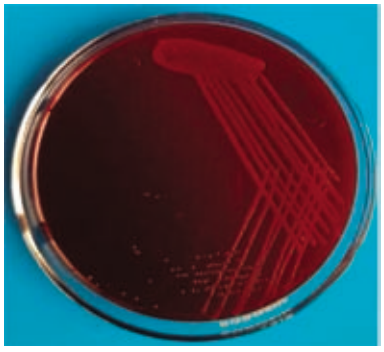

Fig. 5. Inhibition of biofilm by berberine in combination with enrofloxacin

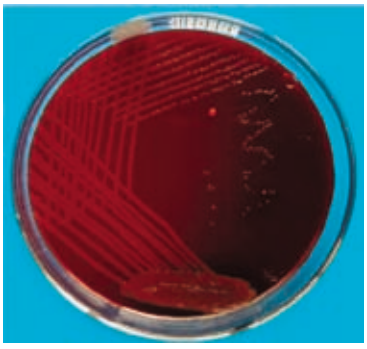

Fig. 6. Inhibition of biofilm by capsaicin in combination with norfloxacin

In the present study, all the isolates were tested positive for biofilm. The formation of biofilm is detected by formation of black dry colonies (Fig. 4). Among the isolates, six isolates were quinolone resistant and six were quinolone sensitive. The isolates that were quinolone sensitive failed to produce biofilm when treated with quinolones alone. The isolates that were quinolone resistant produced biofilm in the presence of quinolones and test compounds when used alone. When the quinolone resistant isolates were treated with combination of quinolone and test compound, the isolates failed to produce biofilm indicating the antibiofilm activity of test compounds in combination with antibiotics. Quinolone resistant *S. aureus* isolates when treated with combination of berberine at concentration above 1g/L when combined with the antibiotics enrofloxacin, norfloxacin and nalidixic acid failed to form biofilm (Fig. 5). Similar results were reported by Liu *et al*., 2015, Li *et al*., 2017 and Shi *et al*., 2018. Berberine inhibits biofilm by inhibiting the amyloid formation or by inhibiting the quorum sensing by the bacteria thus facilitating the entry of antibiotics inside the bacterial cell (Chu *et al*., 2016; Aswathanarayan and Vittal, 2018). Jhanji *et al*. (2020) reported the efflux pump inhibiting activity of berberine to alleviate biofilm formation by bacteria. Inhibition of efflux pumps facilitates the accumulation

of antibiotics inside the bacterial cell hence destroying the bacteria. The reasons for biofilm inhibition by berberine in this study may be the above said mechanisms.

When the *S. aureus* isolates resistant to quinolones were treated with combination of capsaicin above a concentration of 0.5g/L with quinolones like enrofloxacin, norfloxacin and nalidixic acid failed to form biofilm (Fig.6). The result of the study was in accordance with Guo *et al*. (2020). The published report on the antibiofilm activity of capsaicin against *S. aureus* biofilm is scarce. The antioxidant action of capsaicin altering the permeability and oxidative stress within the biofilm can be a reason for antibiofilm activity (Silva *et al*., 2020). Kalia *et al*. (2012) reported the antibiofilm activity of capsaicin in inhibiting the *norA* efflux pump gene conferring resistance to fluoroquinolones. It can be suggested that capsaicin inhibited the NorA efflux pump thus facilitated the accumulation of quinolones inside the bacteria cell.

In the present study, the plant molecules berberine and capsaicin inhibited the biofilm by *S. aureus* isolates from bovine mastitis sample via one or more mechanisms. The number of colonies of bacteria reduced in a dose dependent manner in each combination with the increasing dose of test compounds. Further investigation is needed in this regard to understand the mechanism of action of these compounds in inhibiting biofilm formation with suitable clinical modelling.

Conclusion

The combination of plant molecules with antibiotics can be adopted as a novel treatment strategy in alleviating antimicrobial resistance. Antibiotic sensitivity test revealed the antibacterial activity of berberine and capsaicin in combination with antibiotics. Biofilm assay showed the antibiofilm activity of berberine and capsaicin in combination with quinolones thus enhancing the activity of quinolones. Berberine and capsaicin thus can be plausible drug candidates that can be used as auxillary molecules in treatment. Antibiofilm activity of berberine and capsaicin unveil the scope of further exploitation of plant molecules as suitable drug entrants.

Conflict of interest

The authors declare that they have no conflict of interest.

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Phytochemical analysis and biosynthesis of silver nanoparticles from aqueous extract of seeds of *Sesamum indicum*#

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Abstract

Plants have been in use in medicine from time immemorial. Sesamum indicum, an ancient seed crop, has been widely used mainly for its oil. It is regarded as highly beneficial for its nutritive value and therapeutic effect. It is known to have anti-allergic, anti-inflammatory, anti-cancer, analgesic and many other pharmacological activities. In this study, seeds of S. indicum were subjected to aqueous extraction followed by evaluation for the presence of phytochemical constituents. The aqueous extract of seeds of S. indicum after phytochemical testing, was found to contain alkaloids, phenols, steroids, flavonoids, diterpenes, glycosides and tannins. Following this, silver nanoparticles were biosynthesised from the extract, where the phytochemicals acted as reducing and capping agents for the nanoparticles formed. The synthesised nanoparticles were characterised by using UV-visible spectroscopy, energy dispersive X-ray spectroscopy, field emission scanning electron microscopy and X-ray diffraction. The synthesis of nanoparticles from plant extract is gaining importance now-a-days owing to the effectual activity elicited by them when compared to activity of plant compounds alone.

Keywords: *Sesamum indicum, phytochemicals, silver nanoparticles*

Traditional medicines, which contain compounds derived from medicinal plants, are used by approximately 80 per cent of people in developed countries (Yadav and Agarwal, 2011). Medicinal plants are a valuable source of bioactive compounds with a variety of activities. Secondary plant metabolites (phytochemicals), as they are commonly known, have been linked to a variety

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of biological properties that protect against a range of diseases (Renjith and Sankar, 2020).

Sesame (*Sesamum indicum*), one of the world's important oil crop, is a member of Pedaliaceae family. It is an annual shrub with white bell-shaped flowers with a hint of blue, red or yellow with or without branches. It is grown for the production of seeds that are rich in oil content. These seeds are known to have desirable pharmacological effects. Sesame seeds are proven for their antioxidant activity (Anilakumar *et al*., 2010). The protein from sesame possesses antihyperlipidemic property which reduces triacylglycerol and cholesterol levels in plasma (Biswas *et al*., 2010). The antimicrobial activity of sesame has been reported by Saleem, 2011 against *Salmonella typhi*. Anticancer activity has been observed with myrsitic acid present in sesame seeds (Shasmitha, 2015). Sesamin from sesame has been known to have antihypertensive action in animals (Nakano *et al*., 2006). Miscellaneous effects like antipyretic, anti-inflammatory, wound healing, analgesic activities have been reported (Mushtaq *et al*., 2020).

Irrespective of the pharmacological effects that bioactive plant components possess, their use in the medicinal field has a long way to go. Exhaustive research has been conducted to facilitate nano delivery of these phyto-constituents. These nano formulations are known to have increased bioavailability, solubility, achieving determined target with reduce dose levels (Subramanian *et al*., 2016). Silver nanoparticles play an important role in the field of nanotechnology because of their extraordinary properties including chemical stability, conductivity, catalytic activity and biological effect such as antibacterial (Qais *et al*., 2019), antifungal (Ghojavand *et al*., 2020), antiviral (Sujitha *et al*., 2015), anti-inflammatory (Moldovan *et al*., 2017), anti-oxidant (Keshari *et al*., 2020), anti-plasmodial, anti- diabetic (Kuppusamy *et al*., 2016) and anticancer (Basak *et al*., 2018) activities. They are also known to have larvicidal, acaricidal wound healing properties and used for the treatment of water (Firdhouse and Lalitha, 2015). The present study focused on the production of AgNPs from the aqueous seed extract of *Sesamum indicum* (*S. indicum*) and their characterisation.

Materials and methods

Collection of seeds and identification

The seeds of *S. indicum* were collected from Avalpoondurai, Erode, Tamilnadu (Fig. 1). The plant along with seeds were authenticated from Research and PG Department of Botany St. Thomas College, Thrissur (Fig. 2).

Fig. 1. Seeds of *Sesamum indicum*

Fig. 2. Herbarium

Aqueous extract from seeds of S. indicum

The seeds of *S. indicum* were cleaned, dried and sieved. Then they were crushed and defatted using hexane as a solvent. The defatted residue was then allowed for air drying and kept in Soxhlet apparatus for hot aqueous extraction. Subsequently, it was allowed for drying in the rotary evaporator. The yield of extract was calculated from the extract obtained (Aathira *et al*., 2021). Finally, the resultant product was stored in refrigerator for further study.

Phytochemicals analysis

The secondary metabolites like alkaloids, phenols, steroids, flavonoids, diterpenes, triterpenes and saponins from the aqueous extract of seeds of *S. indicum* was examined as per method described by Harborne (1998).

For detecting alkaloids, one gram of the extract was mixed with 5 mL of ammonia before being extracted with the same volume of chloroform. Five millilitres of dilute hydrochloric acid were added to this extract. The obtained acid layer was then tested for the presence of alkaloids by using various reagents as described below.

Mayer's test - Eight drops of Mayer's reagent were added to 1 mL of acid layer. The presence of alkaloids was indicated by the formation of a cream-colored precipitate.

Dragendorff's test - One mL of acid extract was mixed with eight drops of Dragendorff's reagent. The presence of alkaloids was indicated by the formation of a reddish-brown precipitate.

Hager's test - Eight drops of Hager's reagent were added to 1 mL of acid extract. The presence of alkaloids was indicated by the formation of yellow precipitate.

Wagner's test – Equal amount of extract and Wagner's reagent were mixed together. The presence of alkaloids was indicated by the formation of a reddish-brown precipitate.

The presence of steroids was detected using Salkowski's test, which includes adding 3 mL of chloroform to 50 milligrams of the extract and dissolving it. The solution was then allowed to stand after adding few drops of concentrated sulphuric acid. The presence of steroids was indicated by the formation of a red colour. Also analysed by Liebermann Burchardt test by adding 3 mL of chloroform with 50 mg of extract and mixed. Along the sides of the test tube, five drops of acetic anhydride and one millilitre of concentrated sulfuric acid were added. The presence of steroids was confirmed by the formation of a reddish ring at the junction of two layers.

The detection of glycosides was done by Benedict's test by mixing the extract (50 mg) with 1 mL of water before adding 5 mL of Benedict's reagent. The presence of reducing sugars was indicated by the formation of brown or red precipitate.

The detection of phenols was performed using ferric chloride test by adding five milligrams of the extract to one millilitre of water, dissolving it and adding five drops of 10% ferric chloride. The appearance of a bluish black colour indicated the presence of phenols.

The presence of tannins was confirmed by ferric chloride test by adding two milligrams of extract to three millilitres of one per cent ferric chloride solution. The presence of tannins was indicated by the development of a blue-black or brownish green coloration.

The flavonoids were detected by ferric chloride test by adding four drops of neutral ferric chloride solution to 2 mL of the methanol extract (0.5-gram extract in 10 mL methanol). The presence of flavonoids was indicated by the formation of green colour.

The diterpenes in the extract were detected by adding five milligrams of extract with three millilitres of 5 per cent copper acetate solution. The presence of diterpenes was depicted by the formation of green colour.

The triterpenes were detected using Salkowski test, which includes mixing three milligrams of extract with three millilitres of chloroform and shaken with three millilitres of concentrated sulphuric acid. The presence of triterpenes was indicated by the development of yellow colour in the lower layer after standing.

The presence of saponins were done by froth test by adding 5 mL of water to 200 mg of extract and the tube was shaken. It was identified by the persistence of foam produced for 10 min.

Biosynthesis of silver nanoparticle from aqueous extract

The silver nanoparticles were synthesised by mixing 10 mL of 1 mM silver

nitrate solution with 10 mL of 2 grams of aqueous extract, dissolved in deionised water. It was kept on hot plate and the extract was added under continuous stirring. Then the solution was kept under bright sunlight for the colour change manifesting the development of nanoparticles.

Characterisation of the synthesised silver nanoparticles

The synthesised silver nanoparticles were characterised by UV- visible spectroscopy (Perkin-Elmer, Lamda 25) with wavelength of 300-700 nm. The silver nanoparticles from the plant were examined under field emission scanning electron microscope at Amrita Centre for Nanosciences and Molecular Medicine, Kochi. Using ImageJ software, the size of the nanoparticles was measured.
The energy dispersive X-ray spectroscopy for the biosynthesised silver nanoparticle was performed to detect the presence of specific elements at Amrita Centre for Nanosciences and Molecular Medicine, Kochi.

The phase analysis for the biosynthesised silver nanoparticles was done using X-ray diffraction spectroscopy at Centre for Materials for Electronics Technology (C-MET) Athani, Thrissur. They were verified at 30 kV voltage, 20 mA current at a rate of 10°2θ/ min with CuKα radiation.

Results and discussion

Phytochemical analysis

The yield of the extract was calculated as 13.63 percent**.** Similar extract yield of 13.37 per cent has been reported from aqueous extract of sesame seeds (Hussain *et al*., 2018). The aqueous extract from the seeds showed the presence of steroids, alkaloids, glycosides, flavonoids, phenols, diterpenes and tannins. Phytochemicals analysis by Narasimhan and Mohan (2012) from hexane and methanolic extract of seeds of sesame revealed presence of alkaloids, polyphenols, glycosides, flavanoids, carbohydrates, proteins, phytosterols and terpenoids. Ghani *et al*. (2012) examined the ethanolic defatted extract of *Sesamum indicum* and found to have alkaloids, tannins, phenols, glycosides, saponins, coumarins and flavonoids. These compounds exhibited anticancer activity in Human larynx epidermoid carcinoma (Hep-2 cell line), murine Mammary Adenocarcinoma (AMN-3) and human Rhabdomyosarcoma (RD). This is also in accordance with the work done by Usha *et al*. (2013) from the hot aqueous extract of seeds of *Butea monosperma*.

Biosynthesis of silver nanoparticles from aqueous extract

The manifestation of colour change was analysed by exposing the solution to sunlight that revealed the colour change for yellow to dark red solution (Fig. 3). This indicated the formation of silver nanoparticles. Alfuraydi *et al*. (2019) described similar colour change reaction with silver nanoparticles from sesame oil cake and determined that the transition was caused by surface plasmon resonance and free electron modifications. The aqueous extract containing phytochemicals reduced the silver nitrate to metallic silver and acted as reducing and capping agents for the nanoparticles produced as reported by Basu *et al*. (2016).

Fig. 3. Colour change from yellow to dark red

Characterisation of silver nanoparticles from aqueous extract

The silver nanoparticles from the sesame seeds were analysed using UV-visible spectrophotometer. It showed peak of absorption at 432 nm (Fig. 4). Similar peaks have been observed with Meena and Chouhan (2015). They described that dark red colour was due to the excitation of electrons resulting from the surface plasmon resonance when analysing the fenugreek seed aqueous extract. Azizi *et al*. (2013) also noticed peak at 420 nm with silver

nanoparticles prepared from aqueous extract of *Sargassum muticum*.

The micrographs of FESEM were used to determine the size of the nanoparticle, which was biosynthesised from sesame seeds. They showed a range of 14 - 48 nm in nano scale (Fig. 5). This has been measured using ImageJ software with 64-bit JAVA. The analysis indicated the spherical nanoparticles in solitary and cluster form. This result was similar to consistent silver nanoparticles from *Delphinium denudatum* root produced by Suresh *et al*. (2014), where the FESEM analysis exhibited the nanoparticles in spherical shape. Benakashani *et al*. (2016) also obtained similar spherical nanoparticle of 10 to 40 nm size.

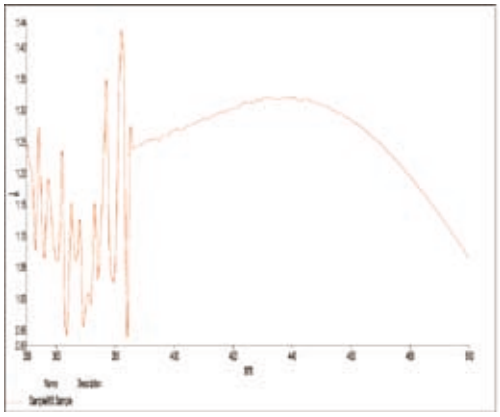

Fig. 4. Absorption spectra for S-AgNPs

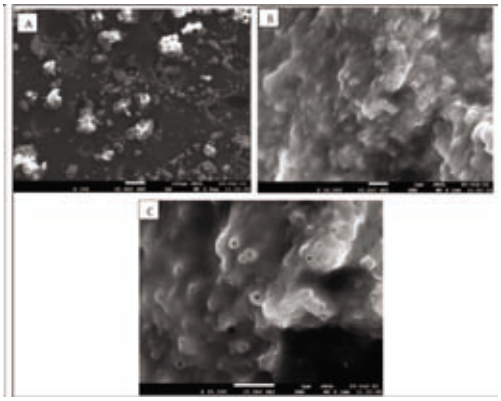

Fig. 5. FESEM images for S-AgNPs- A-100X, B- 10,000 X and C- 20,000 X magnification

With the energy dispersive X-ray spectroscopy, the main peak observed in the graph was silver. Miscellaneous peaks were noticed along the peak of silver. The other peaks were carbon, sulphur, chlorine and oxygen. This might be acquired from the plant extract (Fig. 6 and 7). The findings of this study were in accordance with Bhagat *et al*. (2019).

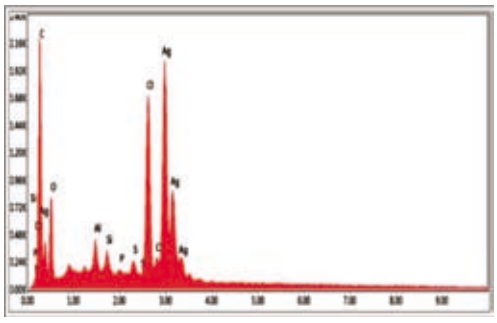

Fig. 6. EDS analysis of S-AgNPs

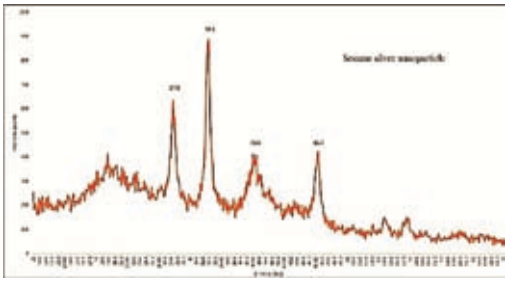

Fig. 7. Diffraction peak for S-AgNPs

With X-ray diffraction analysis, the diffraction analysis for silver nanoparticles produced distinct peaks at 27.8 °, 32.3 °, 38.4 ° and 46.3°. Similar peaks were seen with silver nanoparticles from *S. indicum* oil cake as done by Alfuraydi *et al*. (2019). He also added that those nanoparticles from sesame oil cake were analysed to have better antimicrobial and anti-tumour activity. Miscellaneous peaks with peaks of silver nanoparticles were due to presence of phytochemicals from the seed extract (Ibrahim, 2015).

Conclusion

The aqueous extract from seeds of *S. indicum* analysed for phytochemicals (steroids, alkaloids, glycosides, flavonoids, phenols, diterpenes and tannins) is known to have many pharmacological activities like anti-cancer, anti-inflammatory, anxiolytic, thrombolytic actions. The silver nanoparticles prepared from the aqueous extract was characterised by using UV-visible spectroscopy, energy dispersive X-ray spectroscopy, field emission scanning electron microscopy and X-ray diffraction. Characterisation of nanoparticles is imperative

in revealing their diverse biological activities. These silver nanoparticles biosynthesised from *S. indicum* could be evaluated for several enhanced pharmacological and therapeutic effects that would be beneficial to the population.

Conflict of interest

The authors declare that they have no conflict of interest.

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Assessment of hygienic and sanitation practices among poultry butchers in selected Municipality areas of Assam (India)

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Abstract

A study was conducted in Dhubri and Biswanath Chariali Municipality areas to assess the adoption of hygienic and sanitation practices being followed by poultry butchers. A total of 60 poultry butchers were selected randomly, 30 from each Municipality area so that the final sample consisted of 60 poultry butchers. Data were collected using a pre-tested structured interview schedule by personal interviews. The schedule was designed to collect information on the socio-economic profile of the butchers, personal and meat shop hygiene, maintenance of meat shop and its equipment. The data revealed that all the poultry butchers were male among which (75%) of them had an education level only up to eight standard. The overall mean age of the poultry butchers was found to be 39.95±8.64 years of which majority (75%) of them belong to middle age group. The present study indicated that none of the poultry butchers underwent any formal training for hygienic meat handling. It was also pointed out that most (85%) of the butcheries were located at market area, while only a few (15%) were found in the residential area. Only 18.33 per cent of the poultry butchers wore clean clothes while 81.67 per cent of them did not adopt this practice during working.

Majority (88.33%) of them did not wash their hands after smoking/ chewing tobacco. It was also revealed that majority (88.33%) of the butchers did not clean knives before and after cutting of meat. Majority of the butchers agreed that cleanliness of equipment (71.67%), the meat shop and its surrounding (68.33%) and personal hygiene (68.33%) were some of the important factors that were essential to ensure wholesome meat production. From the above study, it may be concluded that appropriate interventional measures by the concerned agencies such as awareness trainings for poultry butchers on crucial areas of food safety, hygienic practices relating to meat handling and personal safety are imperative. The results of the study also shed light on the need for measures to improve the infrastructural facilities in poultry meat butcheries and for appropriate interventions to strengthen the food quality control system by the government regulatory authorities.

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Diarrhoeal diseases are the most common illnesses resulting from consumption of contaminated food, causing 550 million people to fall ill and 230000 deaths every year in the world (FAO, 2020). Unsafe food creates a vicious cycle of disease and malnutrition, particularly affecting infants, young children, elderly and the sick. Meat is a rich source of various nutrients and is one of the highly perishable food items. Hence, it is highly susceptible to microbial contamination that results in food borne illness among consumers. Microbial contamination also causes spoilage of meat. This can result in quality deterioration with resultant quantity and economic losses in addition to and public health concerns (Komba *et al.* 2012). The types and extent of microbial contamination depend on the extent to which sanitation procedures and hygienic practices are adopted during meat handling, storage, distribution and processing (Ercolini *et al*. 2006; Adu-Gyamfi *et al*., 2012). Failure to adopt good sanitation and hygiene practices such as washing of hands, wearing of protective clothing, cleaning and sanitization of butchery equipment and utensils, transportation of meat in clean containers and storage of meat at appropriately low temperatures can lead to microbial contamination, meat quality deterioration and post-harvest meat losses (Bogere and Baluka., 2014). Moreover, during slaughter, meat and poultry carcasses can become contaminated if they are exposed to even small amounts of intestinal contents. A lack of awareness and the conventional practices followed in processing, handling, and marketing reflect the poor quality of meat. Poor meat hygiene and sanitation may lead to increased risk of food borne illness upon consumption (Gurmu *et al.,* 2013). In many developing countries the fresh meat is primarily distributed through markets or small or medium meat stalls where the hygiene is the least concern. Along with the above issues, knowledge of meat handlers and butchery workers about such hygienic precautions is very poor due to which public are suffering because of the parlous state of meat consumed (Gurmu *et al*., 2013). Due to sluggishness of the concerned authority on licensing, inspection, supervision etc., the hygiene and sanitation status is in a poor condition in Assam. Although there are laws and legislation governing the operations of abattoirs in India, awareness among the butchers with regard to meat hygiene and personal hygiene are far from the desired state of affairs. It was in this context that the present study to assess the personal hygienic practices adopted by butchers, as well as their way of handling and processing meat, perception about maintenance of meat hygiene in the poultry meat stalls of the Municipality areas of Dhubri and Biswanath Charilali (Assam state, India) was carried out.

Materials and methods

The study was conducted in Dhubri and Biswanath Chariali Municipality areas of Dhubri and Biawanath districts of Assam, respectively. Thirty poultry butchers were selected randomly from each municipality area, thus a total of sixty poultry butchers were selected for this study. Before the study, a series of discussion were held with scientists, extension educationists and extension functionaries to develop the interview schedule. After developing the interview schedule draft, it was pre-tested among five butchers in each municipality area to assess the relevancy. Finally, it was mailed to five different experts (judges) of College of Veterinary Science, AAU, Khanapara, who were specialized in this field. After getting response from the experts, certain modifications were made in the interview schedule to make it for final use. Thus, a structured interview schedule was developed containing all relevant information on social profile of the butcher, their experience in butchering, location and structure of shop, license details, awareness towards the personal hygiene, meat borne diseases and meat hygiene. In addition, maintenance of shop and its equipment, the level of personal and meat hygiene maintained during selling of meat, disposal of the poultry wastes and drainage facilities availability were also recorded. The data were collected during the month of June to September, 2021 by personal interviews with poultry butchers. Some of the data were also recorded while visiting the poultry butchers by observation and discussion. The data so collected were coded, classified, tabulated

Table 1. Distribution of respondents based on their socio-economic profile

Parameters	Category	Dhubri (n=30)	Biswanath (n=30)	Total (n=60)
Sex	Male	30	30	60 (100.00)
	Female	0	0	0 (0.00)
Education	Illiterate	2	0	2 (3.33)
	Up to 5th Std.	7	6	13 (21.67)
	6th to 8th Std.	18	12	30 (50.00)
	9th to 12th Std.	3	9	12 (20.00)
	Above 12th Std.	0	3	3 (5.00)
Age (Years)	Young (<30)	6	3	9 (15.00)
	Middle (30-50)	21	24	45 (75.00)
	Old (>50)	3	3	6 (10.00)
	Mean ± SD	38.93 ± 8.92	40.97±8.37	39.95±8.64
Experience as a poultry butcher (Years)	Low (Up to 5)	6	6	12 (20.00)
	Medium (6 to 10)	11	18	29 (48.33)
	High (Above 10)	13	6	19 (31.67)
	Mean ± SD	9.63 ± 4.10	8.2±3.60	8.92±3.88
Any other occupation	Yes	0	2	2 (3.33)
	No	30	28	58 (96.67)
Training attended in meat hygiene	Yes	0	0	0 (0.00)
	No	30	30	60 (100.00)
Do you possess a trade license for a butcher	Yes	20	22	42 (70.00)
	No	10	8	18 (30.00)

and analyzed using the software; Statistical Package for the Social Science (SPSS 16.0). The presentation of data was done to give pertinent, valid and reliable answer to the specific objectives. Frequencies, percentage, mean and standard deviation were worked out for meaningful interpretation.

Results and discussion

Socio-economic profile of poultry meat butcher

It was found that all poultry butchers were male in both Dhubri and Biswanath Chariali Municipality areas, which might be due to male dominated society in the study areas. Gutema *et al.* (2021) also observed that all meat handlers in beef cattle slaughterhouses and retail shops in Bishoftu, Ethiopia were male. Education plays an important role in motivating people towards adopt practices related to cleanliness and hygiene in their day-to-day activities. In the present study the education of majority of the respondents (75%) was only up to eight standard, which indicated that education level of the respondents was very poor (Table 1). The poor educational status of the respondents might be one of reasons for poor knowledge of butchers on proper handling of meat and personal hygiene. Hence proper training on meat handling could improve knowledge about sanitation and hygiene during meat handling. Afnabi *et al.* (2014) indicated that employees with basic level (at least a primary) of education had a good concept of hygiene practices, while bad practices were attributed to illiterate ones. The overall mean age of the poultry butchers was found to be 39.95±8.64 years of which majority (75%) of them belonged to middle age group (Table 1). The overall mean age recorded in the present study indicated that the people who were more energetic and who were the most responsible member of the family engaged in poultry butchering. Salifu and Teye (2006) also reported that butcher operations were quite energy demanding and might involve a lot of travelling to livestock markets. Hence the older men were unable to cope. The overall mean years of experience that the respondents in poultry butchering was recorded as 8.92±3.88 years among whom almost half of the (48.33%) of the butchers had

Table 2. Distribution of respondents based on location and structure of the poultry meat shop

Parameters	Category	Dhubri (n=30)	Biswanath (n=30)	Total (n=60)
Location of the shop	Residential area	4	5	9 (15.00)
	Market area	26	25	51 (85.00)
Structure of the shop	Open (Table with big umbrella)	19	14	33 (55.00)
	Temporary shed with Tarpaulin roof	6	7	13 (21.67)
	Permanent shed inside the market yard	5	9	14 (23.33)
Other facilities				
Had potable water	Yes	8	11	19 (31.67)
	No	22	19	41 (68.33)
Had soap	Yes	7	10	17 (28.33)
	No	23	20	43 (71.67)
Had detergents	Yes	6	3	9 (15.00)
	No	24	27	51 (85.00)
Had disinfectant	Yes	8	12	20 (33.33)
	No	22	18	40 (66.67)
Had adequate lighting	Yes	28	26	54 (90.00)
	No	2	4	6 (10.00)
Had glass cabinet to display carcasses	Yes	2	9	11 (18.33)
	No	28	21	49 (81.67)
Had dustbins for storing wastes	Yes	19	27	46 (76.67)
	No	11	3	14 (23.33)

medium level (6 to 10 years) of experience and slightly less than one third (31.67%) of them had more than 10 years of experience (Table 1). Along with the educational status, experience will also make one more skillful to perform the work more precisely. Reddy *et al*. (2019) also found that in addition to educational status, experience would also reflect the judgment levels of the butchers in understanding the measures that need to be taken. Majority of the respondents had no other occupation except being engaged as poultry butcher, which indicated that this vocation provided them with a secure means of livelihood. Although training is a basic requirement for the personnel working in slaughter house and meat retail shop, the results of the present study indicated that none of the poultry butchers had undergone any formal training in hygienic meat handling. Earlier studies also indicated that considerable proportions of meat processing employees and meat retail shop employees did not receive any basic training on hygienic handling of meat (Haileselassie *et al.*, 2013; Wassie *et al.*, 2017).

The poultry butchers should be trained on food safety issues. The Food and Agriculture Organization (FAO) also recommends the provision of food safety training to food handlers as an important intervention to improve their knowledge and skills (FAO, 2019). Majority (70%) of the poultry butchers had trade license issued from respective Municipal Board, while the rest (30%) did not have any license for engaging in this vocation and this was a major cause of concern.

Location, structure and facilities available in poultry meat butcheries

The present study revealed that most (85%) of the butcheries were located at market areas, while only a few (15%) were found in residential areas (Table 2). Reddy *et al.* (2019) also opined that majority of the butcheries were located in the market area in YSR Kadapa district of Andhra Pradesh. Majority (55%) of the butcheries in this study were open and did not possess a roof. The butcheries studied did not possess the required shelter provisions and were in most cases just a table, meat cutting slab

Table 3. Distribution of respondents based on their personal hygiene at the meat shop

Attributes	Category	Dhubri (n=30)	Biswanath (n=30)	Total (n=60)
Wore clean cloth	Yes	3	8	11 (18.33)
	No	27	22	49 (81.67)
Wore face mask/Head gear/ Hand Gloves/Aprons	Yes	2	5	7 (11.67)
	No	28	25	53 (88.33)
Wore rings	Yes	7	11	18 (30.00)
	No	23	19	42 (70.00)
Washed hands before and after meat handling	Yes	9	12	21 (35.00)
	No	21	18	39 (65.00)
Washed hands after nose blowing	Yes	12	18	30 (50.00)
	No	18	12	30 (50.00)
Washed hands after smoking, chewing tobacco etc.	Yes	3	4	7 (11.67)
	No	27	26	53 (88.33)
Washed hands after visiting toilet	Yes	26	28	54 (90.00)
	No	4	2	6 (10.00)
Washed of hands after handling of money	Yes	1	0	1 (1.67)
	No	29	30	59 (98.33)
Washed hands after touching bins or other objects	Yes	2	3	5 (8.33)
	No	28	27	55 (91.67)
Spitting while working	Yes	19	21	40 (66.67)
	No	11	9	20 (33.33)
Butcher had open cuts on the hands	Yes	3	4	7 (11.67)
	No	27	26	53 (88.33)

etc. and were seldom covered by a temporary umbrella. The results of this study also indicated that 21.67 and 23.33 per cent of the butcheries studied had a temporary shed structure with a tarpaulin roof and permanent shed structure inside the market yard respectively. The present study indicated that none of the poultry meat butcheries had the requisite organized building structures. According to Food Standard and Safety Authority of India (FSSAI) (2018), the floor of butcheries should be hard, impervious and washable, non-slippery and made of non-toxic materials, without crevices and should be easy to clean and sufficient slope to allow adequate drainage. Similarly, walls should be made of impervious materials, smooth and without crevices for easy cleaning and sanitation and to avoid accumulation/absorption of dust, blood/meat particles, and microbial/ fungal growth. In absence of organized building structures, meat for sale at the butcheries were displayed openly without any protective covering and were thus exposed to dust particles and domestic flies posing the risk of every chance of microbial contamination led to spoilage of meat. The unorganized structures of the poultry meat butcheries might be due to lack of awareness of the butchers. The FSSAI is the supreme authority, which is responsible for regulating and supervising the food safety. So, it is mandatory to take FSSAI license as per the law. Most (68.33%) of the butcheries did not have potable water. Only 28.33 per cent of the butcheries had a provision for soap while in the rest (71.67%) soap was not made available for the use of butchers (Table 2). Tuneer and Madhavi (2015) revealed that majority of the butchers used only water for the purpose of washing, while only a few of them used soap with water. They further observed that most of the butchers used the same water for the whole day continuously for washing and processing purposes. They also reported that none of the workers in slaughterhouses wore aprons nor covered their hairs. Similarly, in the present study only 15 per cent of the butcheries had detergents while majority (85%) of the butcheries had no detergents for washing of equipment. Two-third of the butcheries had no disinfectants. This indicated that most of the butcheries were in unhygienic conditions, which might be due to lack of awareness of

Table 4. Distribution of respondents based on meat shop hygiene and maintenance of equipment

Attributes	Category	Dhubri (n=30)	Biswanath (n=30)	Total (n=60)
Means to control flies and mosquitoes	Yes	7	11	18(30.00)
	No	23	19	42 (70.00)
Disposal of wastes	Nearby open space	19	20	39 (65.00)
	Municipal sewer	11	10	21 (35.00)
	Soak pit	0	0	0 (0.00)
Presence of stray animals, wild birds etc.	Yes	28	27	55 (91.67)
	No	2	3	5 (8.33)
Had clean and rust-free knives	Yes	26	27	53 (88.33)
	No	4	3	7 (11.67)
Had clean cutting slab	Yes	17	20	37 (61.67)
	No	13	10	23 (38.33)
Had clean cage	Yes	6	10	16 (26.67)
	No	24	20	44 (73.33)
Washing of knives before and after cutting of meat	Yes	4	3	7 (11.67)
	No	26	27	53 (88.33)
Used soap and clean water to wash equipment	Yes	5	7	12 (20.00)
	No	25	23	48 (80.00)
Had clean surrounding	Yes	8	10	18 (30.00)
	No	22	20	42 (70.00)

Table 5. Distribution of respondents based on the slaughtering practices

Attributes	Category	Dhubri (n=30)	Biswanath (n=30)	Total (n=60)
Slaughter method	Halal method	25	4	29 (48.33)
	Stunning method	5	26	31 (51.67)
Ensured complete drainage of blood from the carcass	Yes	9	2	11 (18.33)
	No	21	28	49 (81.67)
Blood was collected to prevent environmental pollution	Yes	2	1	3 (5.00)
	No	28	29	57 (95.00)
Blood was spread over the carcass	Yes	29	30	59 (98.33)
	No	1	0	1 (1.67)
Washed carcass before and after evisceration	Yes	10	5	15 (25.00)
	No	20	25	45 (75.00)
Removed viscera immediately after killing the bird	Yes	7	8	15 (25.00)
	No	23	22	45 (75.00)

the respondents. Majority (90%) of the poultry meat shops had adequate lighting facility (Table 2). Displaying carcasses under open sky might expose to dust resulting in deterioration of meat quality. Most (76.67%) of the shops were equipped with waste bins to store wastes before being permanently disposed off.

Personal hygiene of poultry meat butchers

Only 18.33 per cent of the poultry butchers wore clean cloth while 81.67 per cent of did not wear clean cloth during their job in the study areas (Table 3). Majority (88.33%) of the respondents did not wear protective clothes (face mask, head gear, hand gloves and aprons), and only a few (11.67%) of them wore such protective clothes. Wearing clean clothes and other protective clothes could be the good practices at personal level to produce superior quality meat.

The use of gloves by butchers is a significant hygienic measure that could protect the meat against contamination (Alhaji and

Table 6. Perception of butchers regarding maintenance of meat hygiene

Attributes	Category	Dhubri (n=30)	Biswanath (n=30)	Total (n=60)
Selection of healthy birds is important for hygienic meat production	Yes	26	27	53 (88.33)
	No	4	3	7 (11.67)
Cleanliness of the equipment is important for hygienic meat production	Yes	24	19	43 (71.67)
	No	6	11	17 (28.33)
Cleanliness of the meat shop and surrounding is important for hygienic meat production	Yes	20	21	41 (68.33)
	No	10	9	19 (31.67)
Personal hygiene is important for hygienic meat production	Yes	19	22	41 (68.33)
	No	11	8	19 (31.67)
Aware of the diseases that are transmitted through butchering or eating meat	Yes	2	4	6 (10.00)
	No	28	26	54 (90.00)
Aware of occupational hazards/ diseases from birds	Yes	0	2	2 (3.33)
	No	30	28	58 (96.67)
Refrigerator is required to store meat to prevent spoilage	Yes	23	19	42 (70.00)
	No	7	11	18 (30.00)
Spread of blood over meat is safe for hygienic meat production	Yes	26	29	55 (91.67)
	No	4	1	5 (8.33)

Bewai, 2015). Most (70%) of the butchers did not wear rings. Almost two-third of the respondents did not wash their hands before and after meat handling, the while rest of them did so. It was also found that half of the poultry meat butchers washed their hands after blowing their noses. Majority (88.33%) of them did not wash their hands after smoking, chewing tobacco *etc*. However, 90 per cent of the respondents washed their hands after visiting the restroom. The results of the study also shed light on the fact that most (98.33%) of the respondents did not wash their hands after handling money. Majority (91.67%) of the poultry meat butchers chewed tobacco during meat handling. Spitting was a common practice among the poultry meat butchers in the present study and two-third of respondents reported resorting to spitting during meat handling. Some of the respondents had open cuts on their hands which would also pose risks to meat contamination besides placing the butcher at risks of contracting various meat borne infections. All the practices investigated in this discussion such as the handling of meat without washing hands, washing of hands before and after meat handling and after nose blowing are crucial to ensuring optimum personal hygiene among butchers and are pre-requisites for safe and wholesome meat production. Various factors such as ignorance or lack awareness among the poultry meat butchers could have contributed to this phenomenon that places the health of consumers at risk. Appropriate intervention by the concerned authorities in this regard through trainings and motivational exercises are crucial in ensuring that the health of customers and consumers and not compromised. It is imperative that constructive action through appropriate interventions such as trainings and awareness building programmes are forthcoming in this regard. Upadhayaya and Ghimire (2020) also observed that most of the meat handlers practiced smoking, eating or drinking while handling meat. Furthermore, majority of them were found wearing jewellery during meat handling. Jewellery was a potential source of micro-organisms, because the skin under the jewellery provided a favourable habitat for contaminating microorganisms to proliferate (Trickett, 1997).

Maintenance of hygiene of meat shop and equipment

In the present study it was found that the carcasses were displayed in the open without any protective cover and such a process could provide fertile ground for attracting mosquitoes and flies causing nuisance and compromising meat quality as well. Majority (70%) of the butcheries had no measures to control flies and mosquitoes (Table 4). Moreover, it was revealed that most of the poultry meat butchers did not

follow proper methods for waste disposal. About two-thirds of them threw poultry feathers and poultry meat shop wastes in the nearby open space, which is a major cause of concern from the public health point of view. Stray animals such as dog and wild birds like crow were very common around the poultry meat shops under study. Stray animals in the vicinity of a majority (91.67%) of the butcheries was a routine phenomenon in the present study (Table 4), all of which could be a fall out of improper waste disposal procedures followed by the butchers. Majority (88.33%) of the poultry meat shops had clean and rust-free knives for cutting meat into pieces. Likewise, 61.67 per cent of the butcheries had clean cutting slab. Only 26.67 per cent poultry meat shops had clean cage for keeping live birds. It was revealed that majority (88.33%) of the butchers did not clean knives before and after cutting of meat.

Most (80%) of the respondents did not wash equipment with soap and clean water, and they mostly used same water repeatedly to wash their equipment. The surroundings of the poultry meat butcheries in the study areas were mostly found dirty. The unhygienic surrounding of the poultry meat butcheries might be due to improper disposal of poultry waste.

Slaughtering practices of poultry meat butcheries

Halal method of slaughtering was followed by 48.33 per cent, while 51.67 per cent of the respondents followed stunning method for slaughtering of chicken (Table 5). Most (81.67%) of the butchers did not ensure complete drainage of blood from the carcass, which might be due to lack of awareness while, only few (18.33%) ensured complete drainage of blood from the carcass. Complete drainage of blood from carcass could improve the shelf life of meat.

Majority (98.33%) of the respondents spread blood over the carcasses to apparently give an “attractive look” to the meat. However, blood smearing over carcasses could spoil meat due to microbial contamination. Majority (75%) of the butchers did not wash the carcass before and after evisceration. In most (75%) of the cases, it was observed that butchers did not remove viscera immediately after killing the bird.

Perception of butchers about hygienic meat production

Majority (88.33%) of the respondents believed that selection of healthy birds was important for hygienic meat production (Table 6). Majority of the butchers agreed that cleanliness of equipment (71.67%), meat shop and its surrounding (68.33%) and personal hygiene (68.33%) were some of the important factors responsible for wholesome meat production. Most (90.00%) of the respondents were not aware of the fact that diseases that were spread through the process of slaughter or through consuming meat. This might be due to lack of awareness among the butchers under present study which calls for appropriate awareness building exercises. Majority (96.67%) of the butchers were not aware of occupational hazards/ disease from birds (Table 6).

Most (70%) of the butchers studied were aware that refrigerator was required to store meat to prevent microbial spoilage. However, none of the shops could afford the cost of a refrigerator. Devaru *et al*. (2017) also found that the awareness level of the butchers on the health hazards of eating contaminated poultry meat was poor. They also reported that knowledge on the occupational hazards among butchers was low. They further revealed that the most important factors for maintaining hygiene were cleanliness of the shop and cleanliness of the equipment. Present study also revealed that personal hygiene, selection of a healthy bird and disposal of by-products were some of the other factors identified by the butchers to maintain meat hygiene. None of the poultry butchers used any protective equipment like aprons, gloves and gumboots in the present study.

Conclusion

The present study revealed that there were significant lacunae with respect to the adoption of crucial hygienic meat handling practices among the poultry meat butchers which could be instrumental in leading to higher chances of contamination and cross-

contamination of meat with serious implications for human health. The adoption of such unhygienic handling practices could serve as suitable pathways for meat borne pathogens to enter the food chain. Moreover, lack of awareness among poultry meat butchers about personal hygiene and proper meat handling would further help in multiplication of pathogens in meat. Hence the butchers should be trained properly on food safety to improve hygienic meat handling practices along the poultry supply chain. Improvement of infrastructural facilities and financial assistance in the form of subsidies for ensuring maintenance of cold chain on the meat shops along with measures to strengthen the food quality control system by the government regulatory authorities are also required to ensure that this system operates as per international and national standards. Series of awareness and training programmes should be conducted to improve the knowledge of the butchers on hygienic meat production and handling would further help to provide a pavement for the production of wholesome meat.

Conflict of interest

The authors declare that they have no conflict of interest.

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Molecular characterisation of virulence genes in *Staphylococcus aureus* associated with clinical bovine mastitis

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Abstract

Staphylococcus aureus is the most frequently isolated pathogen from bovine mastitis including subclinical, clinical, and chronic infections. Virulence factors possessed by S. aureus aid in causing infection and inflammation by producing toxins and proteins, which are responsible for the pathogenesis of the disease. In the current study out of 51 animals presented with clinical mastitis, S. aureus was isolated from the milk of 18 animals. S. aureus was confirmed by genus and species level identification using polymerase chain reaction. Molecular characterization of selected virulence genes including thermonuclease (nuc) and Panton Valentine Leucocidin (PVL) was performed in all the S. aureus isolates. Presence of nuc gene was observed in all the isolates (100 %) of S. aureus. No isolates were found to be positive for the presence of PVL gene. Profiling the virulence genes is an important tool for epidemiological studies of mastitis, which can be employed for the prevention and control of the disease.

Keywords: *Mastitis, Staphylococcus aureus, virulence genes*

Mastitis can be defined as a complex disease which involves interaction between the host anatomy and physiology, different causative pathogens and environmental factors such as animal husbandry, hygiene and sanitation. In dairy industry worldwide, *S. aureus* is said to be the most common causative organism responsible for bovine mastitis (Miles *et al*., 1992). The infected quarter of affected cows is considered to be the main reservoir for *S. aureus* infection in the herd. The different factors like evasion of immune mechanism of host, invasion and infection of host tissue, spread of bacteria and acquisition of the required nutrients by the pathogenic organism like *S. aureus* could be attributed to its virulence factors (Haveri *et al.,* 2005). The *nuc* gene is

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said to be ubiquitous in all organisms belonging to genus *Staphylococcus*. It is said that regardless of the thermonuclease activity all *Staphylococcus* spp., except *S. scuiri*, possess *nuc* gene (Sasaki *et* al., 2007). Panton Valentine Leucocidin is a cytotoxin, which causes disruption of cell membranes creating pores on polymorphonuclear neutrophils (Kaneko and Kameo 2004). Panton Valentine Leucocidin positive *S. aureus* was found to be responsible for causing mastitis in many countries and it was observed that even though PVL acts strongly on human polymorphonuclear cells, weak activity was observed on bovine neutrophils too (Peton and Le Loir, 2014).

The study of the important virulence factors aids in the identification of their role in bovine mastitis. Each specific virulence factors have role in each stage and type of infection, and it is observed that not all strains of *S. aureus* possess the same virulence factors. Hence, study of virulence factors in *S. aureus* is useful in molecular epidemiological studies of bovine clonal types for the effective prevention and control of mastitis (Fitzgerald *et al*., 2000). Hence, this study was envisaged for the molecular detection of virulence genes, *nuc* and *PVL* from clinical mastitis caused by *S. aureus*.

Materials and methods

Milk samples were collected from 51 animals affected with clinical mastitis and subjected to identification of organisms by morphological characterization, colony characteristics on selective media and biochemical reactions (Barrow and Feltham, 1993; Quinn *et al*., 2013). Isolates presumptive of *S. aureus* were further subjected for confirmation by molecular characterisation. The DNA extraction was performed by snap chill method or heat lysis method as described by Vijayakumar and Jose (2021). All the isolates of *S. aureus* obtained in the study were subjected to genotypic characterisation by amplification of 16S rRNA and 23S rRNA genes for molecular confirmation of *Staphylococcus* spp. and *S. aureus* respectively. The presence of selected virulent factor genes *viz. nuc* for thermonuclease and *PVL* for Panton Valentine Leucocidin were determined by polymerase chain reaction (PCR) using specific primers as shown in Table 1. The reagents and chemicals used for the PCR were Emerald Amp Fast PCR master mix (2X PCR Smart mix, Takara, Japan) forward and reverse primer set (100nM/ml, Sigma Aldrich) and sterile nuclease free water. All the primers were reconstituted in sterile nuclease free water to a final concentration of 10 pmol/µl and stored at -20 °C.

The PCR were performed using the programmable S1000 Thermal cycler, BioRad, USA. Polymerase chain reaction was performed in a total volume of 25µl reaction mixture by combining the reagents. The PCR conditions were optimized by setting different time temperature combinations for annealing processes (Tables 2, 3). The combination that gave the best result for amplification was selected for carrying out further PCR.

After completion of PCR, amplified products were subjected to submarine agarose gel electrophoresis.

Results and discussion

In the present study, out of the samples from 51 animals affected with clinical mastitis, 35 samples yielded growth and a total 38 bacterial isolates were obtained wherein, three samples yielded two different types of growth. Out of the 38 organisms isolated during this study, 27 were contagious pathogens (71.05 %) which included 18 isolates of *S*. *aureus* (47.37 %), 6 isolates of coagulase negative staphylococci (15.79 %) and 3 isolates of *Micrococcus* spp. (7.89 %). The environmental pathogens (28.95 %) that were isolated included 11 coliforms, out of which 6 isolates were *E. coli* (15.79 %) and 5 isolates were *Klebsiella* spp. (13.16 %). *Staphylococcus aureus* was isolated as the major pathogen from bovine mastitis cases and the result was in accordance with the findings by Verma *et al*. (2018), and Workineh *et al*. (2002) who reported the prevalence of *S. aureus* as 42.55 per cent and 40.5 per cent, respectively. Studies conducted on bovine clinical mastitis by Rathish *et al.* (2015) in Thrissur district also revealed *S. aureus* to be the most common pathogen isolated. The present study did not concur with the findings of Fadlelmula *et al.* (2009), who found lower prevalence of *S. aureus*

Table 1. Details of primers used in PCR

Organism / Virulence genes	Genes	Primer sequence	Amplicon size (bp)	Reference
Staphylococcus spp.	*16S rRNA*	F: AACTCTGTTATTAGGGAAGAA CA	756	Ciftci *et al*. (2009)
		R: CCACCTTCCTCCGGTTTGTCACC		
Staphylococcus aureus	*23S rRNA*	F: GGA CGA CAT TAG ACG AAT CA	1318	El - Razik *et al*. (2010)
		R: CGG GCA CCT ATT TTC TAT CT		
Thermonuclease	*nuc*	F: GCCAAGCCTTGACGAACTAAAGC	279	Brakstad *et al.* (1992)
		R: GCGATTGATGGTGATACGGTT		
Panton Valentine Leucocidin	*PVL*	F: GCTGGACAAAACTTCTTGGAATAT	85	Pajic *et al.* (2014)
		R:GATAGGACACCAATAAATTCTGGATTG		

Table 2. PCR protocol for the amplification for characterization of *S. aureus*

Sl. No	PCR Programme		Temperature – Time Protocol	
			16*S rRNA*	**23*S rRNA***
1.	Initial Denaturation		94 °C for 5 min	94 °C for 5 min
2.	Denaturation	30 cycles	94 °C for 45 sec	94 °C for 45 sec
3.	Annealing		56.9 °C for 45 sec	55.8 °C for 45 sec
4.	Extension		72 °C for 90 sec	72 °C for 90 sec
5.	Final extension		72 °C for 10 min	72 °C for 10 min
6.	Hold		4 °C Until use	4 °C Until use

Table 3. PCR protocol for the amplification of virulence genes of *S. aureus*

Sl. No	PCR Programme		Temperature - Time Protocol	
			nuc	**PVL**
1.	Initial Denaturation		94 °C for 5 min	95 °C for 5 min
2.	Denaturation	30 cycles	94 °Cfor 1 min	95 °C for 30 sec
3.	Annealing		55 °C for 30 sec	59 °C for 30 sec
4.	Extension		72 °C for 90 sec	72 °C for 60 sec
5.	Final extension		72 °C for 3.5 min	72 °C for 5 min
6.	Hold		4 °C Until use	4 °C Until use

of 9.8 per cent in their study. Jose *et al.* (2021) reported in their studies a lower prevalence of coliforms, *Klebsiella* spp. (7.40 per cent) and *E. coli* (4.47 per cent), isolated from clinical bovine mastitis which was consistent with our study.

Hence, from clinical bovine mastitis cases, 18 isolates of *S. aureus* were identified by biochemical methods and subjected to molecular confirmation by polymerase chain reaction after DNA extraction by snap chill method. This method was used for extraction of bacterial DNA from cases of bovine mastitis by many researchers (Shah *et al*., 2020; Nazir *et al.,* 2017). Lange *et al*. (2015) performed species level identification of *Staphylococci* and concluded that *16S rRNA* sequencing is an accurate method for the identification of staphylococci from bovine mastitis. But due to the lack of heterogenicity in *16S rRNA* gene, it would be difficult to identify and discriminate different *Staphylococcus* species (Petti *et al*., 2008).

Stephan *et al.* (2001) used *23S rRNA* for the species level identification of *S. aureus* from bovine mastitis, and all the 34 isolates could be identified as *S. aureus.* Hence, all the bacterial isolates presumed to be *Staphylococcus* spp. were subjected to PCR by targeting *16S rRNA* and for the species level identification of the isolates, *23S rRNA* was targeted. From clinical bovine mastitis, 18 isolates were identified and confirmed as *S. aureus* respectively (Fig. 1 and Fig. 2).

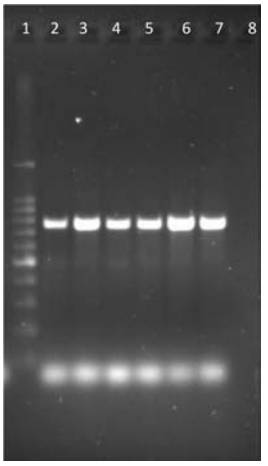

Fig. 1. Agarose gel electrophoresis of *16S rRNA* specific PCR of *Staphylococci* spp.
Lane 1 – Ladder
Lane 7 – Positive control
Lane 8 – Negative control
Lane 2,3,4,5,6,7 – Positive samples (756 bp)

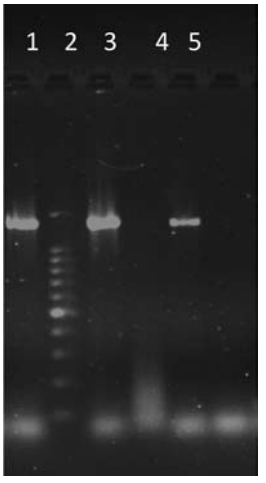

Fig. 2. Agarose gel electrophoresis of *23S rRNA* specific PCR of *S. aureus*
Lane 1,3 – Positive samples (1318 bp)
Lane 2 – Ladder
Lane 4 – Negative control
Lane 5 – Positive control

The virulence factors thermonuclease and Panton Von Leukocidin coded by *nuc* and *PVL* respectively were screened. The *nuc* gene is commonly employed for the species level identification of *S. aureus*. The positive amplicon size of 279 bp for *nuc* gene was detected in all the 18 isolates (100 per cent) of *S. aureus* (Fig.3). This was in accordance with many studies wherein, *nuc* gene was used for

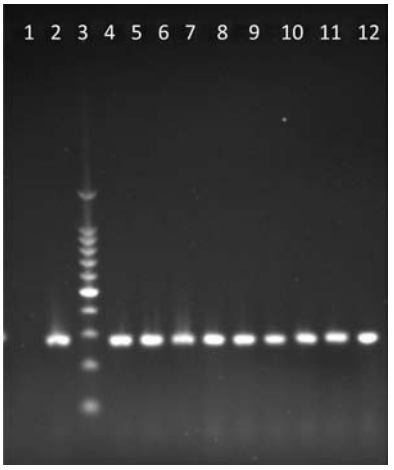

Fig. 3. Agarose electrophoresis of *nuc* gene specific PCR of *S. aureus*
Lane 1 – Negative control
Lane 2 - Positive control
Lane 3 – Ladder
Lane 4,5,6,7,8,9,10,11,12 – Positive samples (279 bp)

the identification of *S. aureus* organism isolated from bovine mastitis (Ciftci *et al*., 2009). *S. aureus* possess *nuc* gene which has species specific sequences which on amplification have the potential for detection as well as identification of *S. aureus* from infections (Brakstad *et al*., 1992; Costa *et al.,* 2004).

Panton Valentine Leucocidin is a phage encoded virulence factor which has major public health significance. Screening was performed for the presence of *PVL* in 18 isolates of *S. aureus* from bovine mastitis and none of them was found to have the presence of the gene. Similarly, absence of *PVL* was observed in other studies (Patel *et al*., 2021; Prashanth *et al*., 2011). Varying occurrence of *PVL* in bovine mastitis is observed throughout India, as high as 41.6 per cent (Mitra *et al*., 2013) and as low as 10.53 per cent (Shrivastava *et al*., 2018). Identification of *PVL* in bovine isolates is a rare finding and the frequency observed in other studies on bovine mastitis was attributed to the contamination of milk by milkermen carrying *PVL* containing *S. aureus* isolates (Fluit, 2011, Shrivastava *et al*., 2018). Unlike other leukotoxins, PVL is found to have weak action on bovine neutrophils, and this could be the probable reason for the absence of *PVL* in bovine strains and for its presence in human strains of *S. aureus* (Prevost *et al.,* 1995).

Conclusion

The present study concluded that there is presence of virulence gene in *S. aureus* associated with bovine mastitis isolated from Thrissur district. Further study of different virulence genes in a large study population must be conducted for extrapolating the data, to use it for epidemiological investigation and for the prevention and control of mastitis caused by *S. aureus.*

Conflict of interest

The authors declare that they have no conflict of interest.

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Effect of supplementation of biotin in total mixed ration of dairy cows on rumen fermentation characteristics by *in vitro* gas production technique

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Abstract

An experiment was conducted to assess the effect of supplementation of biotin on rumen fermentation parameters and microbial biomass production by in vitro gas production technique. Biotin was supplemented to the substrate - total mixed ration (TMR) at various doses viz., T1-0 (control), T2-0.5, T3-1.0, T4-1.5 and T5-2.0 mg/kg DM. The in vitro true dry matter degradability, total gas production, metabolizable energy and microbial protein production were found be increased proportionally to the dose of biotin. However, the methane and volatile fatty acid production were not affected by biotin supplementation. Results revealed that the positive effects on in vitro ruminal fermentation were dose-dependent and biotin can be incorporated in the diet of dairy cows to improve nutrient digestibility and rumen biomass production.

Keywords: *TMR, rumen fermentation characteristics, microbial biomass, biotin*

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Biotin is an important water-soluble vitamin in the diet of both monogastric and ruminant animals. Biotin is naturally present in plants and also synthesized in the rumen by microbes. It plays an important role in a number of microbial metabolic processes, including gluconeogenesis and lipogenesis (Said, 2012). Dairy cows reared intensively may have a higher need for biotin. Various reports suggested that biotin synthesis was reduced by approximately 50 per cent when the ratio of concentrate to roughage increased. (Da Costa Gomez *et al*.,1998). Similarly, increasing the concentrate portion also resulted in decreased ruminal biotin synthesis (Abel *et al*., 2001). This suggests that high-concentrate diets fed to high-producing dairy cows can negatively influence net biotin synthesis in the rumen, due to the acidic conditions in the rumen, and this may aggravate the need for supplemental biotin. B complex vitamins are generally not supplemented to ruminants, due to the fact that rumen microbes have the ability to synthesise B complex vitamins in the rumen.

Rumen microbes convert inferior feed ingredients into high-quality microbial proteins to meet the nutritional needs of ruminants. This microbial protein supplies 60 to 85 % of amino acids reaching the animal's small intestine (Hristov, 2007; Owens *et al*., 2009; Krizsan *et al*., 2010). In the current scenario, measures to augment the efficiency of feed utilisation and production are essential to improve the economic viability of livestock farming. Feeding total mixed ration (TMR) will improve feed efficiency in animals because each mouthful of feed that the cow consumes contains balanced amount of nutrients. It will provide stable environment in the rumen for the activity of rumen microbes. It also gives energy and nitrogen sources at adequate level for optimum production of microbial protein and volatile fatty acids (Mackawa *et al.*, 2002). The supplementation dose of vitamin B for ruminants is not optimized. Hence, this experiment was intended to find the optimum dose of biotin for effective rumen biomass production.

Materials and methods

The *in vitro* gas production studies were carried out using Hohenheim gas production technique (Menke and Steingass, 1988). The biotin was supplemented at different levels (T1, T2, T3, T4 and T5 - 0, 0.5, 1.0, 1.5 and 2.0 mg/kg DM respectively) of the basal diet. The basal diets comprised paddy straw and concentrate mixture in 29:71 ratio. Feed samples were analysed for proximate principles (AOAC, 2016). Neutral detergent fibre (NDF) and acid detergent fibre (ADF) were determined using the method described by Van Soest *et al.* (1991).

To carry out *in vitro* gas production technique (IVGPT), rumen liquor was collected from early lactating crossbred cows fed on standard TMR in accordance with ICAR (2013) using stomach pump. The rumen liquor was strained through four layers of cheese cloth, transferred into pre-warmed CO_2-filled thermos flask. The temperature of the rumen fluid was maintained at 39°C throughout the preparation of the incubation medium. Fermentation was conducted in 100 ml glass syringe. The syringes were prewarmed (39°C) for 1-hour, before the addition of 30 ml of buffered rumen fluid into each syringe under CO_2 flushing. Three blank syringes containing only 30 ml of buffered rumen fluid were incubated to estimate gas production due to endogenous substrates for the blank corrections. 200 mg of TMRs were fortified with different levels of biotin and were incubated in 30 ml of incubation medium. The syringes were then placed in automatic shaker water bath incubator at 39°C. Analyses were completed in six replicates of each treatment and trial run in twice with readings of gas production recorded after incubation for 0 and 24 hours. The fermented fluid was collected for the estimation of volatile fatty acids, *in vitro* true dry matter degradability and *in vitro* true organic matter degradability (Banakar *et al*., 2017). Metabolizable energy and microbial protein was calculated from the data as per Blummel and Lebzien (2001).

Total gas production

Gas produced (ml/ 200 mg substrate) by fermentation of substrate feed over a 24-hour period was measured after correcting corresponding blank values.

In vitro true DM and OM digestibility

Goering and Van Soest (1970) method were followed for the determination of true dry matter digestibility (TDMD) and true organic matter digestibility (TOMD).

$$\text{TDMD \%} = \frac{(\text{DM taken for incubation} - \text{NDF residue}) \times 100}{(\text{DM taken for incubation})}$$

$$\text{TOMD \%} = \frac{(\text{OM taken for incubation} - \text{OM residue}) \times 100}{(\text{OM taken for incubation})}$$

Where, DM – Dry matter, OM – Organic matter, NDF – Neutral detergent fibre

Microbial biomass production

Microbial biomass production (MBP) of the TMR tested was calculated from TDOM using the equation

MBP (mg) = TDOM (mg) – (Corrected gas production for 24 hrs × 2.20)

Where 2.20 is the stoichiometric factor for roughages (Blummel *et al*., 1997) and for mixed diets (Blummel and Lebzien, 2001)

TDOM- Truly digested organic matter

Metabolizable energy (ME)

ME of target TMR was calculated by formula given by Menke and Stiengass (1988)

ME (KJ/kg DM) = 1.24+ 0.146 × gas (ml / 200mg DM) + 0.007× CP + 0.0224× EE

Where, CP - Crude protein, EE - Ether extract

Methane estimation

The percentage of methane production was estimated by collecting and injecting the gas produced on *in vitro* study into the Methane Gas Analyser (0-100%; Precision Equipment Private Limited) at Central Instrumentation Laboratory, CVAS, Mannuthy. **(**Purushothaman *et al.,* 2019; Sadan *et al*., 2019 and Neenu, 2021).

Estimation of volatile fatty acids

Volatile fatty acid composition of the inoculum was estimated using 7890A NUCON 5700 gas chromatograph, as per standard procedure described by Filipek and Dvorak (2009). On completion of the incubation, the buffered rumen liquor was filtered through four layers of muslin cloth and approximately 0.8 mL of the sample was preserved with 200 µl of 25 per cent metaphosphoric acid and allowed to stand for half an hour, then centrifuged at 7000 rpm for 20 min at 4°C. The samples preserved in this way were immediately analysed or stored at -20°C for future analysis.

Observations made on the various parameters *viz*. true dry matter digestibility

Table 1. Ingredient composition of total mixed ration used in *IVGPT*

Ingredient	Quantity (parts per quintal)
Maize	23
Rice polish	10
Corn gluten fibre	10
De-oiled rice bran	10
Alfalfa pellet	8
Coconut oil cake	8
Paddy straw	29
Mineral mixture	1
Salt	1
Total	100

Table 2. Chemical composition of TMR[1] used in IVGPT (% DM basis)

Parameter	Nutrient composition (%)
Dry matter	92.63 ± 0.41
Crude protein	13.28 ± 0.12
Ether extract	3.74 ± 0.10
Crude fibre	12.55 ± 0.12
Total ash	11.45 ± 0.12
Nitrogen free extract	58.99 ± 0.27
Acid insoluble ash	5.11 ± 0.1
Calcium	0.90 ± 0.02
Phosphorus	0.56 ± 0.02
Neutral detergent fibre	35.25 ± 0.19
Acid detergent fibre	24.74 ± 0.24

[1]Mean values are based on six replicates with S.E.

Table 3. *In vitro* gas production and fermentation parameters of TMR supplemented with biotin in cross bred cows

Treatments	Total gas (mL)	CH4(%)	ME (MJ/kg DM)	TDMD (%)	TOMD (%)	MBP (mg)
T1	28.54± 0.35^{a}	19.25 ± 0.23	5.61 ± 0.05^{a}	67.39 ± 0.16^{a}	70.43 ± 0.55^{a}	63.36 ± 1.52^{a}
T2	30.25± 0.18^{b}	19.08± 0.15	5.86 ± 0.03^{b}	73.2 ± 0.31^{b}	74.96 ± 0.72^{b}	67.55 ± 1.38^{b}
T3	33.02± 0.20^{c}	18.84± 0.25	6.27 ± 0.03^{c}	75.28 ± 0.3^{c}	79.65 ± 0.25^{c}	69.86 ± 0.66^{bc}
T4	35.23± 0.25^{d}	18.72± 0.19	6.59 ± 0.04^{d}	77.42 ± 0.27^{d}	83.27 ± 0.48^{e}	71.59 ± 0.98^{c}
T5	35.93± 0.20^{d}	18.80± 0.34	6.7 ± 0.04^{d}	77.86 ± 0.21^{d}	81.4 ± 0.35^{d}	66.88 ± 0.97^{b}
F-value	159.53	0.822	159.53	273.86	110.34	7.49
P-value	0.001**	0.523^{ns}	0.001**	0.001**	0.001**	0.001**

[1]Mean values are based on six replicates with S.E.

**Mean± S.E. of different treatment having different alphabets as superscripts within a column differ significantly at p<0.01

NS- Non-Significant

Table 4. Volatile fatty acid concentration[1] of TMR supplemented with biotin in cross bred cows assessed *in vitro*

Treatments	Acetic acid (mMol/L)	Propionic acid (mMol/L)	Butyric acid (mMol/L)	Total volatile fatty acids (mMol/L)	Acetate: propionate ratio
T1	46.77±0.29	19.04±0.52	5.28±0.16	71.09±0.30	2.47±0.08
T2	46.12±0.74	19.08±0.58	6.16±0.10	71.36±0.97	2.43±0.08
T3	46.71±0.41	19.94±0.57	5.42±0.21	72.08±0.65	2.35±0.07
T4	45.94±0.26	20.59±0.51	5.73±0.29	72.26±0.89	2.24±0.05
T5	45.91±0.16	19.77±0.45	5.68±0.51	71.36±0.25	2.33±0.05
F- value	1.024	1.484	1.341	0.563	1.692
P- value	0.414^{ns}	0.237^{ns}	0.282^{ns}	0.691^{ns}	0.183^{ns}

[1]Mean values are based on six replicates with S.E.

NS – Non-Significant

(TDMD) and true organic matter digestibility (TOMD), microbial biomass production, metabolizable energy and methane production were subjected to cluster analysis and based on this, the best level of biotin was identified.

Data gathered on the various parameters were analysed statistically as per Snedecor and Cochran (1994) by analysis of variance (ANOVA) technique, using the statistical software, SPSS - version 24.0 (IBM Corp., 2016)

Results and discussion

Proximate composition

The chemical composition and fiber fractions of the evaluated samples are presented in Table 2. The CP, EE, CF, total ash, NFE, AIA, calcium, phosphorus, NDF and ADF contents of the basal substrate were found to be 13.28±0.12, 3.74±0.10, 12.55±0.12, 11.45±0.12, 58.99±0.27, 5.11±0.1, 0.90±0.02, 0.56±0.02, 35.25±0.19 and 24.74±0.24, on DM basis, respectively.

In vitro gas production

Ruminal fermentability characteristics evaluated by *in vitro* gas production are depicted in Table 3. In biotin supplemented TMRs the gas production for 24 hours ranged from 28.54±0.35 to 35.93±0.20 mL. Biotin supplementation significantly increased the amount of gas produced during the first twenty-four hours. Methane varied from 18.72 ± 0.19 to 19.25 ± 0.23 %, which was found

to be similar among the groups. Contrary to the present findings, Poolthajit *et al.* (2021) reported increased methane production in TMR supplemented with combination of betaine, biotin and chromium picolinate @3 g/kg DM and 6 g/kg DM, but it had no effect at high levels - 9 g/kg DM assessed by IVGPT.

Digestibility and metabolizable energy

Metabolisable energy values ranged from 5.61 ± 0.05 to 6.7 ± 0.04. The corresponding TDMD % ranged from 67.39 ± 0.16 to 77.86 ± 0.21, the TOMD % from 70.43 ± 0.55 to 81.4 ± 0.35. Supplementation of biotin to basal diet significantly ($P < 0.01$) improved ME, the *in vitro* TDMD and TOMD (Table 3). Higher level of supplementation showed significantly higher *in vitro* TDMD and TOMD (biotin 1.5 mg/kg DM level). Increase in nutrient digestibility due to supplementation may be because of the stimulation of rumen microbial growth.

In accordance with present results, Cruyvagen and Bunge (2004) also reported an improved fermentability and NDF digestibility in cows which were supplemented with biotin. Kandathil and Bandla (2019) reported that fortification of the basal substrate with biotin increased the total gas production in Deoni cows. They reported that B-vitamin synthesised by one microbe is consumed by others. As there is no absorption of B vitamins in rumen, the supplementation of B vitamins through feed could augment rumen biomass production. On the contrary, Grewal *et al*. (2016) reported that supplementation of biotin @ 1.33 mg/kg DM of complete feed had no effect on the net gas production, digestibility of true OM, NDF and ME availability.

Microbial biomass production

The MBP (mg/200mg DM) calculated from the gas production and TOMD data are listed in Table 3. MBP (mg) production ranged from 63.36 ± 1.52 to 71.59 ± 0.98 respectively. The statistical analysis of the data on MBP (mg/200mg DM) revealed a significant difference ($P < 0.01$) among the treatments. Microbial nitrogen is the major source of protein for ruminants and is utilized to meet the maintenance requirement of the animal. It was highest in T4 and is indicative of a good amount of fermentable substrate aiding the growth and development of rumen microorganism. Gas production, ME, TDMD and TDMD was highest in T4 and T5, whereas MBP was highest in T4.

Biotin supplementation in this trial possibly stimulated cellulolytic bacteria as they depend on other microbes for their metabolic biotin requirements (Baldwin and Allison, 1983). Where biotin was omitted from an *in vitro* medium, Milligan *et al.* (1967) observed a decrease in fibre digestion. They postulated that the biotin deficiency caused a blockage of one or more steps in the propionate production pathway resulting in a depletion of vital intermediates, which in turn resulted in reduced rates of cellulose digestion.

Volatile fatty acids production

Data related to total volatile fatty acids (TVFA; mmol/L), acetic acid, propionic acid, butyric acid and acetate: propionate ratio is presented in Table 4. Statistical analysis of the data revealed that there was no significant difference ($P > 0.05$) in the TVFA and individual fatty acids among the groups. Similarly, Zimmerly and Weiss (2001) reported that supplementation of dietary biotin (0, 10, or 20 mg/day) had no effect on the molar percentages of the ruminal volatile fatty acids Holstein cows. Suksombat *et al*. (2011) also reported that incorporating biotin supplements at the rate of 0, 20 and 40 mg/cow/day in Holstein Friesian dairy cows had similar concentration of volatile fatty acids among the groups.

Conclusion

The study revealed that there was major variation in *in vitro* rumen fermentation parameters with addition of biotin. Supplementation of biotin for manipulating rumen fermentation was effective and showed positive results on *in vitro* true dry matter degradability, *in vitro* organic matter digestibility, ME and microbial biomass production. It could be inferred that biotin supplementation @ 1.5 mg/kg DM can be used for optimizing the rumen fermentation characteristics in dairy cows. In future *in vivo* research to be done to validate the results.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Carcass characteristics and proximate composition of Mithun (*Bos frontalis*) carcass

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Abstract

In order to study the carcass characteristics, six mithun (Bos frontalis) of either sex were slaughtered and their body tissues were analysed for proximate principles in the present investigation. The physical carcass characteristics like the carcass length, weight, dressing percentage etc. were also determined. The data analysis revealed that the mean carcass length and weight were measured as 162.10±6.15 cm and 242.25±36.75 kg, respectively. The overall dressing percentage was found to be 60.65±1.68% in mithun, while the subcutaneous fat thickness was recorded to be 42.15±2.15 mm. The heaviest organ of the mithun carcass was observed to be the stomach (68.2±0.1 kg), the lightest was recorded to be the pancreas (0.45±0.10 kg). The nutrient composition of mithun meat was excellent with protein content of 18.74±1.25% and fat content of 0.48±0.03%. This study further recommended the need of a detailed study on the effect of mithun meat consumption on human health in future.

Keywords: *Mithun, carcass traits, proximate composition*

Mithun (*Bos frontalis*) is a unique bovine of the northeastern hills (NEH) region of India and adjacent areas of Bhutan, Bangladesh, China and Myanmar. Apart from its cultural attachment to the tribal society of the region, mithuns have predominantly been used for meat purpose. The multi-faceted contribution of this massive semi-domesticated bovine has a special role in the overall improvement of socio-economic condition of the mithun eating countries (Mondal and Pal, 1999). Though specific and more systematic research works have been taken for nutritional, anatomical, physiological and ethological aspect of mithun production, there are only some sporadic reports on the meat production by this species (Heli *et al.*, 1994; Pal *et al.*, 2002; Chungath and Kima, 2018). Therefore, the present study was undertaken to study the physical characteristics of the mithun carcass and to find out the proximate composition of mithun meat.

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Materials and methods

Six apparently healthy young mithuns, aged between 2 and 3 years of either sex, were slaughtered at the Medziphema Mithun Farm, National Research Centre on Mithun, Jharnapani, Nagaland, India following traditional method (Heli *et al.*, 1994). The different physical characteristics like carcass length, fresh carcass weight, mean blood volume, fat weight and thickness, loin muscle area (measured by conventional formula for beef animals as per Pinto (1988) and Osório and Osório (2005)), weights of both edible and non-edible offals were recorded immediately. The weights of various parts and organs were taken with the help of a weighing balance having an accuracy upto 0.050 kg. The four chambers of stomach were weighed with their contents as the consumers in Nagaland usually take them as edible offals after removing the content. The biometrical measurements of the organs were also done during the study with the help of ordinary plastic measuring tape having a graduation upto 0.100 cm.

To find out the proximate composition, different body tissues like muscle, liver, kidney, heart, intestine, bone, skin and blood were aseptically and hygienically collected (50 g each) from the slaughtered animals and were immediately brought to the laboratory for their further analysis for chemical composition. The samples were cut into pieces in the laboratory and then dried in hot air oven at 100.0 ± 5.0°C overnight (except blood) to determine moisture content, crude protein, ash, crude fat, and NFE as per the standard procedures (AOAC, 2016). Blood samples were collected at the rate of 100 ml per carcass and subjected to analytical procedures. Blood samples were separately dried under steam and then analysed. The data analysis was done following suitable statistical procedures (Snedecor and Cochran, 1994).

Results and discussion

The fresh carcass weight was recorded to be 242.25±36.75 kg, while the average carcass length was measured as 162.10±6.15 cm (Table 1). The overall dressing percentage in mithun carcass was found to be 60.56±1.68%, the loin muscle area being 716.50±65.50 sq. cm. The present findings corroborated with the earlier reports (Heli *et al.*, 1994). However, the average carcass weight in case of buffaloes (*Bubalus bubalis*) and cattle (*Bos taurus*) were comparatively less (Naveena and Kiran, 2014) than that of mithun (*Bos frontalis*) reported in the present study. This may be attributed to the feeds and feeding management under which mithuns were being kept as well as species difference in growth and the age of the animal at which slaughtered. The dressing percentage in conventional beef producing buffaloes (Naveena and Kiran, 2014; Lambertz *et al*., 2014; Tamburano *et al*., 2019; Kumar *et al*., 2020) was also much lower than the observed value in our study. Overall, the physical characteristics of the carcass justified its being used as a potential meat animal in its natural habitat area. The mean blood volume collected from mithun carcass was 4.23 per cent of the total carcass weight.

Various visceral and non-visceral organs of mithun were measured for their biometry and the results were presented in Table 2. It was revealed that the stomachs (4 chambers) with content constituted about 28.15 per cent of the total carcass weight. Among the edible offals of the mithun carcass, the parts of four limbs weighed the most (68.2±0.1kg) and the pancreas, the least (0.45±0.10 kg). The present findings were in line with the reported values for buffaloes (Anjaneyulu *et al.,* 1985, 2007).

The proximate composition of the body tissues of the mithun was analysed and presented in Table 3. It indicated average protein content (fresh basis) of 18.74±1.25% in this red meat having fat content of 0.48±0.02%. The highest fat in the mithun carcass was found in bone tissues (3.58±0.95%) and the highest protein content was in its skin (24.89±2.08%). Moisture percentage of 74.04 to 77.75 per cent has been reported for fresh buffalo meat (Anjaneyulu *et al.,* 1985; Naveena *et al.,* 2004). The protein content of mithun meat in the present study was higher than the previous workers who reported 17.90 per cent crude protein content on fresh basis (Anonymous, 2001). Buffalo meat showed a protein percentage of 17.33 to 23.3 per cent (Naveena *et al.,* 2004). Among all the red meats, buffalo has been

Table 1. Physical characteristics of mithun carcass

Parameters	Average values (n=6)
1. General	
Fresh carcass weight (kg)	242.25±36.75
Carcass length (cm)	162.10±6.15
Dressing percentage (%)	60.56±1.68
Weight of subcutaneous fat (g)	416.85±6.85
Blood volume (%)	4.43
Back Fat thickness (mm)	42.15±2.15
Loin muscle area (sq cm)	716.50±65.50
2. Weight of edible offal (% of fresh carcass weight)	
Heart	0.70
Kidney	0.41
Liver	1.94
Stomachs (with contents)	28.15
Intestines	11.82
Parts of limbs (include legs, head, claws etc.)	37.81
3. Weight of non-edible offal (% of fresh carcass weight)	
Lungs	1.20
Pancreas	0.19
Spleen	0.33
Horns	1.94
Hooves	1.84
Skin	16.43

reported to have lowest concentration of total lipids (1.37g/100g) and buffalo meat from 2 year old male calves showed a fat percentage of 1.0 to 3.5 (Kesava Rao and Kowale, 1991). Our present findings showed that mithun meat is much leaner than other animal species and the relatively low fat content in mithun meat is attributed to poor marbling.

The present findings were in agreement with the earlier reports on the composition of mithun carcass (Heli *et al.*, 1994). Several studies on related species like cattle and buffaloes also showed that the values reported in our study were of similar trend as far as the nutrient composition of the carcass is concerned (Lambertz *et al*., 2014; Naveena and Kiran, 2014).

Conclusion

The physical characteristics of mithun carcass and chemical composition of its various body parts recorded in the present study suggest its suitability for human consumption. The present report recommends a further detailed study on the Mithun carcass characteristics for its effect on human health. However, the fatty acid profile and other meat quality parameters including collagen content, mineral content and organoleptic quality may be taken up in future for advising nutritious animal food source in mithun eating countries.

Table 2. Biometry of some visceral and non-visceral organs of Mithun

Body organs	Mean values (cm) (n=6)	
	Length	Breadth
Tongue	38.07±2.35	6.35±0.52
Larynx	0.55±0.12	0.32±0.09
Pharynx	0.75±0.10	0.20±0.01
Lungs	53.00±4.97	22.32±1.98
Heart	19.95±2.46	17.37±2.12
Liver	47.50±3.56	27.75±2.43
Spleen	46.75±3.15	14.20±1.09
Stomachs: Rumen	89.62±7.58	80.82±10.95
Reticulum	27.57±5.64	18.00±3.16
Omasum	36.75±6.29	20.50±3.51
Abomasum	49.60±6.23	25.50±4.26
Intestines: Small	36.72±5.27	2.52±0.92
Large	8.50±1.27	5.00±0.79
Testicle	10.60±1.53	5.10±1.04
Ovary	9.25±1.05	3.55±0.65

Table 3. Mean (±SE) of chemical composition (on fresh basis) of different body tissues of Mithun (n=12)

Nutrient (%)	Muscle	Liver	Kidney	Heart	Intestine	Blood	Bone	Skin
Moisture	78.82±0.53	74.73±1.38	83.14±2.61	80.01±0.96	79.59±2.53	85.46±3.45	38.87±2.38	71.14±1.32
DM	21.18±0.08	25.27±1.04	16.86±1.25	19.99±0.98	20.41±1.96	14.54±1.87	61.13±3.16	28.86±1.56
OM	20.04±1.21	22.80±1.95	14.22±1.00	19.50±1.03	19.83±1.49	13.72±1.69	40.86±2.25	28.21±2.11
Ash	1.11±0.20	1.66±0.28	1.19±0.08	0.95±0.01	0.94±0.08	0.81±0.04	33.11±3.45	0.64±0.03
CP	18.74±1.25	18.11±2.35	12.82±1.68	15.47±1.00	15.92±2.31	12.60±2.56	18.35±1.97	24.89±2.08
Ether Extract	0.48±0.03	1.59±0.53	1.47±0.06	1.72±0.30	1.08±0.59	0.09±0.00	3.58±0.95	0.40±0.05
NFE	1.03±0.74	3.89±0.01	1.50±0.27	0.88±0.07	2.46±1.00	1.32±0.84	5.87±1.24	0.00±0.00

Conflict of interest

All the authors have declared that they have no conflict of interest.

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A scale to measure knowledge level of dairy farmers affected by Kerala flood 2018 on disaster response

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Abstract

Kerala was worst affected by flood disaster in August 2018. The most effective strategy to mitigate the adverse effects of flood disaster is providing the general public with basic knowledge of how to respond to disasters. The present study was contemplated to develop and standardise a scale for measuring dairy farmers' knowledge on disaster response. Based on thinking and differentiation of well knowledgeable dairy farmers from poorly knowledgeable dairy farmers 27 items comprehensively covering each aspect of disaster response was constructed. Based on relevancy test, 16 items were selected. These selected knowledge items were subjected to item analysis comprising of difficulty index, discrimination index and point biserial co- relation. A total of 9 items were selected for the final scale. The reliability of the knowledge test was measured by Cronbach alpha. Cronbach's alpha was found to be excellent .811, which is very high and indicates strong internal consistency among the 09 items. The developed knowledge test was found to be highly stable and dependable measurement.

Keywords: *Disaster, response, knowledge scale, reliability, validity.*

Knowledge is a highly valued state in which a person is in cognitive contact with reality. Knowledge is a relation, on one side of the relation is a conscious subject, and on the other side

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is a portion of reality to which the knower is directly or indirectly related. (Zagzebski, 2017). The knowledge and skills are derived from man's daily interactions with the environment, observations and experiments. (Lenka and Satpathy, 2020). Disasters are defined as sudden unforeseen events with natural, technological or social causes that lead to destruction, loss and damage (Alexander, 2005). India has been traditionally vulnerable to natural disasters on account of its unique geo-climatic conditions. Among the flood-affected nations, India is known as one of the severely flood-affected countries in Asia, with one-fifth of global flood deaths and 12 per cent of geographical areas prone to various kinds of floods. (Mohanty *et al.,* 2020).

In August 2018, the state of Kerala experienced its worst flooding since 1924. As per IMD data, Kerala received 2346.6 mm of rainfall from 1 June 2018 to 19 August 2018 in contrast to an expected 1649.5 mm of rainfall which was about 42 per cent above the normal. (CWC, 2018).This unusual rainfall resulted in the most devastating floods of the century. The flooding occurred as a result of extreme rainfall in a short period of time, the geographical uniqueness of Kerala's land pattern and lack of quality drainage system (Saravanan *et al.,* 2021). Union of India declared this flooding as Level 3 calamity "calamity of severe nature". The state witnessed a loss of 400 human lives along with the loss of nearly 12,000 dairy animals and the destruction of about 57,000 hectares of cultivated land (Sachin *et al.,* 2022). The estimated economic loss was more than $3.8 million (Hunt and Menon, 2020).

One of the key gaps as observed in Kerala post disaster needs assessment (PDNA) Floods and landslides – August 2018 by United Nations was that the flood warning is understood by people but was ignored. Hence, community preparedness to respond to the flood was low. Although the flood warnings were provided to the community, there was reluctance to respond to warnings due to lack of knowledge about the impact of the flood. Further, the report recommended for improving knowledge, innovative measures and appropriate use of technology to address the flood situation (United Nations, 2018).

The agriculture and animal husbandry sectors are most vulnerable to disaster fury. According to Behera *et al.* (2020) floods results in unavailability of feed and shelter, affecting livestock in a stressed condition and ultimately suppressing their immunity, making them susceptible to infectious diseases. Li *et al.* (2013) contemplated that the most effective strategy to mitigate the adverse effects of a disaster is providing the general public with basic knowledge of how to respond to disasters (disaster response knowledge).

In this context, it is prudent that the dairy farmers are made knowledgeable about the methods to overcome flood and other disasters. Accordingly, a knowledge test was developed to assess dairy farmer's knowledge on disaster response keeping in mind the vulnerability of the Kerala dairy farmers to flood disaster. There is no proper scale available to measure dairy farmers' knowledge on disaster response. Hence, the present study was contemplated to develop and standardise a scale for measuring dairy farmers' knowledge on disaster response.

Materials and methods

In the present study, knowledge was operationalised as the information and understanding of the dairy farmer regarding disaster response. The knowledge test was developed and standardised by employing the following procedure

Item collection and relevancy rating

The content of knowledge test was composed of questions (items). An item pool of questions was prepared by reviewing the literature, referring textbooks and conducting discussions with subject matter specialists and field extension personnel. The questions were designed to test the knowledge level of flood-affected dairy farmers about disaster response. A total of 27 knowledge items were initially constructed for the relevancy test. The selected statements were subjected to scrutiny by an expert panel of judges to determine the relevancy and screening for inclusion in the

final scale as per the method suggested by Kumar and Ratnakar (2016). The statements satisfying the following criteria i.e., relevancy percentage >70, relevancy weightage >0.70 and mean relevancy score > 2.8 were selected. A total of 16 items were selected.

Item analysis

Item analysis is used for creating a viable question bank and to assess the respondent's performance as a part of formative assessment. All the items collected for the construction of the knowledge test were in the objective form. The questions were yes or no items involving impersonal and objective assessment. The 16 questions selected were subjected to sixty respondents who were flood affected dairy farmers. The investigation was conducted in two panchayats *viz.,* Kozhinjampara and Perumatty gram panchayats in Palakkad district, Kerala during December 2020 and the duration of study was 60 days. Initially, the base data of flood affected dairy farmers in the two panchayat were collected from panchayat office, Kerala state Animal Husbandry Department and Department of Dairy Development. A total of thirty flood affected dairy farmers from each panchayat were randomly selected.

For each correct answer, one mark was assigned. For each wrong answer as well as those which the respondents don't know as scored as zero. The respondents' total knowledge score was calculated by summating the scores of all the questions. The calculated knowledge scores were used to calculate difficulty index, discrimination index and point biserial correlation.

Difficulty index (DI)

Difficulty index (p-value), also called ease index, describes the percentage of respondents who correctly answered the item. It ranges from 0 – 100 per cent. The higher the percentage, the easier the item. The recommended range of difficulty is from 25 – 75 per cent. Items having p-values below 25 per cent and above 75 per cent are considered difficult and easy items respectively. (Hingorjo and Jaleel, 2012). The difficulty index of each of the 16 items was calculated dividing the total correct responses for a particular item by the total number of respondents as under

$$Pi = \frac{ni}{Ni} \times 100$$

where,

Pi = difficulty index in percentage of the i^{th} item
ni = number of respondents giving correct answer to i^{th} item
Ni = total number of respondents to whom the items were administered i.e. 60

Discrimination index (DcI)

The item discrimination value of an item indicates the degree to which a single item predicts the value of the item battery. It is the ability of each individual item to discriminate between respondents with different levels of knowledge by measuring its correlation score on each item with the overall test score. The difficulty index values range from -1 to 1. The higher the value, the better the item measures what is intended to measure (Priyadharshini *et al.*, 2021). The statement which is answered correctly by everyone or the one which is not answered by anyone in the sample had no discrimination value. Therefore, only those statements with high power to discriminate the respondents who varied in the level of knowledge were included in the final list. The discrimination power of all the 17 items was worked out using E1/3 method to find out the item discrimination, as given below. In this method, the 60 respondents were divided into six equal groups, each having ten respondents and they were arranged in descending order of the magnitude of their knowledge scores as obtained from them. The middle two groups were eliminated. Only four extremes groups i.e. the groups with highest and lowest scores were considered to calculate the 'Discrimination Index'. It is calculated by the following formula.

$$E1/3 = \frac{(S1+S2) - (S5+S6)}{N/3}$$

where,

N = Total number of respondents to whom the items were administered.

S1 and S2 are the frequencies of correct answers

Table 1. Item analysis

Sl. No	Knowledge items	DI	DcI	Rp-bis value
1	During the event of flood disaster , dairy animals have better chance of survival if they are untethered*	71.67	0.3	0.384
2	During flood disaster move the dairy animals to higher ground *	63.33	0.3	0.341
3	Animals are natural swimmers*	68.33	0.3	0.353
4	The foremost important step to be taken in response to flood disaster is evacuation*	60	0.45	0.423
5	108 is Nationwide emergency contact number in case of any emergency*	70	0.25	0.287
6	101 is the phone number to be contacted in case of Fire	85	0.45	0.575
7	The Kerala Disaster Response Force is stationed at Peermedu, Idukki District	25	0.3	0.247
8	The regional response Centre of National Disaster Response Force is stationed at Kozhikode	11.67	0.35	0.411
9	Advice about care and management of animals during disaster can be accessed from veterinary department*	78.33	0.3	0.417
10	The nearest NDRF unit for Kerala is stationed at Arakonam - CISF	13.33	0.4	0.448
11	Name any voluntary organisations in your locality involved in disaster response (Name any one)*	70	0.45	0.495
12	During flood one should move in still water , not in moving water*	61.67	0.55	0.406
13	Walking in six inches of moving water will be dangerous	86.67	0.35	0.325
14	Kerala state emergency operations centre phone number is +91 471-236 4424	11.67	0.35	0.415
15	During cyclones the animals are safer outside than those sheltered	16.67	0.5	0.491
16	Deceased dairy animals should be disposed by deep burial*	53.33	0.45	0.408

*Statements selected for knowledge test

DI – Difficulty index DcI – Discrimination index Rp-bis - Point biserial correlation

of highest and higher scores, respectively

S5 and S6 are the frequencies of correct answers of lower and lowest scores, respectively

Point biserial correlation (Rpbis)

The main aim of calculating point biserial correlation (Rpbis) is to work out the internal consistency of the items i.e., the relationship of the total score to a dichotomized answer to any given item. It is the correlation between right/wrong scores obtained by respondents on a given set of items. It is a special type of correlation between a dichotomous variable (the multiple-choice item score which is right or wrong, 0 or 1) and a continuous variable (the total score on the test ranging from 0 to the maximum number of multiple-choice items on the test) (Sureshverma *et al.,* 2018). The point biserial correlation is calculated by

$$Rpbis = \frac{Mp-Mq}{Sigma} X\sqrt{pq}$$

where,

Rpbis is the point biserial correlation,

Mp is the mean of the total score of the respondents who answered an item correctly

Mq is the mean of the total score of the respondents who answered an item incorrectly, sigma is the standard deviation of the entire sample,

p is the proportion of the respondents giving correct answer to an item

q is the proportion of the respondents giving incorrect answer to an item.

The calculated point biserial correlation values were statistically tested with n-2 degrees of freedom.

Results and discussion

The items, having difficulty index value within 0.25 to 0.75 and discrimination index value above 0.2 were selected for preparation of the final scale according to methodology adopted by Kumar *et al.* (2016). Along with the above selection criteria those items which secured point bi serial correlation value which was significant at 5 per cent level of significance were selected for the final items of the knowledge test. Thus, finally, 09 items (Table 1) were selected for the knowledge test which was considered as neither too difficult nor too easy to reply to and could discriminate the well-informed individuals from the less-informed ones.

Validity of the knowledge test

The validity of a scale is defined as "the extent to which an instrument measures the latent dimension or construct it was developed to evaluate" (Chan *et al.,* 2021). The validity of the knowledge test was established through content validity. Content validity refers to the adequacy with which a measure assesses the domain of interest. The need for content adequacy is vital if the items are to measure what they are presumed to measure. The content validity of the knowledge test was ensured by choosing items in consultation with various subject matter specialists. All possible care was taken while selecting the items and the same was subjected to difficulty and discrimination index and point biserial correlation, to select the final statements. Hence, it was logical to assume that the test satisfied representative as well as a sensible approach of test construction, the criteria for content validity.

Reliability of the knowledge test

Reliability is defined as consistency in results from repeated measurements(Louangrath, 2018). The reliability of the test was determined by the Cronbach alpha coefficient of reliability test. The selected knowledge items were administered to 40 flood affected dairy farmers who were selected randomly from two panchayats viz., Kozhinjampara and Permatty in Palakkad district, Kerala during December 2020. The collected data were tabulated and analysed to estimate the alpha value. The alpha was calculated using formula as follows

$$\alpha = \frac{K}{K-1}\left(\frac{\sum_{i=1}^{K} \sigma^2 y_i}{\sigma^2 x}\right)$$

Where,

α = Cronbach's alpha reliability coefficient,
K = Number of items,
$\sigma^2 y_i$ = the variance of item i for the current sample of persons,
$\sigma^2 x$ = the variance of the observed total test scores.

Cronbach's alpha was found to be excellent (0.811), which is very high and indicates strong internal consistency among the 09 items. Essentially, this means that respondents who tended to select high scores for one item also tended to select high scores for the others; similarly, respondents who selected a low score for one item tended to select low scores for the other knowledge statements. Thus, knowing the score for one knowledge statement would enable one to predict with some accuracy the possible scores for the other knowledge statements.

Table 2 highlights the column containing the 'Corrected item-total Correlation' for each of the items. It indicates the correlation between a given knowledge item and the sum score of the remaining items. The table also highlights the Cronbach's alpha that would result if a given item was deleted. It also shows the alpha value if the given item was not included among a set of items. For example, for Item1, if it was deleted the Cronbach's alpha would drop from the overall total of .811 to .808. It explains that the alpha would drop with the removal of the first knowledge statement (Item1), which appears to be useful and contribute to the overall reliability of the knowledge scale.

Taber (2018) surmised that alpha values were described as excellent (0.93–0.94), strong (0.91–0.93), reliable (0.84–0.90), robust (0.81), fairly high (0.76–0.95), high (0.73–0.95), good (0.71–0.91), relatively high (0.70– 0.77), slightly low (0.68), reasonable (0.67–0.87), adequate (0.64–0.85), moderate (0.61– 0.65), satisfactory (0.58–0.97), acceptable (0.45–0.98), sufficient

Table 2. Item total statistics

Item	Scale mean if Item Deleted	Scale Variance if Item Deleted	Corrected Item - Total Correlation	Cronbach's Alpha if Item Deleted
Item 1	1.83	6.240	0.378	0.808
Item 2	1.82	6.219	0.377	0.808
Item 3	1.85	6.528	0.271	0.819
Item 4	1.80	5.600	0.641	0.775
Item 5	1.80	5.978	0.464	0.798
Item 6	1.83	5.840	0.568	0.785
Item 7	1.80	5.198	0.833	0.747
Item 8	1.84	5.859	0.575	0.784
Item 9	1.81	5.998	0.469	0.797

(0.45–0.96), not satisfactory (0.4–0.55) and low (0.11). In present developed knowledge scale, the alpha value was found to be reliable, which indicates the strong internal consistency among the set of items.

Conclusion

Items selected for knowledge test fall within the range of recommended difficulty index, discrimination index, reliability and validity to ensure correct measurement of knowledge on disaster response. The test so developed could be used for assessing the knowledge level of dairy farmers on disaster response. Based on the knowledge levels the strategies could be chalked out for implementing disaster mitigation activities. This scale can be used to measure the farmers' knowledge on disaster response beyond the study area with suitable modifications. Before application of this knowledge test to broader category of farmers in other vocations a need based analysis to understand the actual problem faced, need to be undertaken to understand.

Conflict of Interest

Certified that there is no conflict of interest to be declared in the present work.

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Ameliorative efficacy of polyherbal formulation in streptozotocin induced diabetic rats

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Abstract

The aim of the present study was to determine the anti-diabetic activity of a polyherbal formulation in streptozotocin induced diabetic rats. The methanolic extracts of Achyranthes aspera, Catharanthus roseus and Momordica charantia were used in 3:1:1 w/w/w ratio for polyherbal formulation (PHF) preparation. Fifty Sprague-Dawley rats were randomly divided into five groups (GI, GII, GIII, T1 and T2) based on body weight. The high fat diet treated rats of four groups (GII, GIII, T1 and T2) were injected with 45 mg/kg body weight of streptozotocin intra peritoneally to induce diabetes. Rats of group GI, GII were kept as normal and diabetic control respectively and GIII as standard control, were administrated with glibenclamide (2.5 mg/kg, orally for 60 days); and group T1 and T2 were administered with PHF daily at a dose of 375 mg/kg and 750 mg/kg body weight respectively for a period of 60 days. Administration of PHF showed dose dependent reduction in the blood glucose level on 30th and 60th days, which was comparable to that of standard drug glibenclamide. Significant decrease in body weight and increase in serum glucose level were observed in diabetic control, which was partially restored upon administration of PHF. Altered biochemical enzymes like AST, ALT and antioxidant enzymes (SOD) were normalized by administration of PHF in diabetic rats. Also, mean values of total cholesterol, triglyceride, HDL-cholesterol and LDL-cholesterol in rats treated with PHF were restored. The study indicated that PHF at higher dose showed significant decline ($p<0.001$) in blood glucose level.

Keywords: *Polyherbal formulation, Streptozotocin, type-2 diabetes.*

Diabetes mellitus is a group of chronic metabolic abnormalities, caused by the loss of glucose homeostasis due to improper insulin secretion or action or both resulting into defect in glucose metabolism and other energy-yielding fuels such as lipids and proteins (Moller, 2001). In diabetes, glucose in the blood fails to enter cells, thereby increasing the blood glucose level.

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Diabetes is a major healthcare problem, associated with nerves and blood vessels damage, leading to complications such as heart disease, stroke, kidney dysfunction, blindness, nerve problems, gum infections etc (Frances and Patrik, 2012). Chronic ailments occur mainly due to imbalance pro-oxidants and antioxidants in the organism, leading to oxidative stress (Tayyab and Lal, 2016) and is also an important cause of progression of β-cell dysfunction, insulin resistance, impaired glucose tolerance and type 2 diabetes mellitus (Wright *et al.,*2006).

At present, diabetes is mainly treated by the use of biguanides, thiazolidinediones, sulfonylurea, D-phenylalanine derivatives and α-glucosidase inhibitors besides insulin. However, because of side-effects the efficacies of these compounds are arguable and there is a demand for new compounds / molecules with good therapeutic potential and less adverse effects for the treatment of diabetes (U.K. prospective diabetes study16, 1995).

Medicinal plants are treasured supply of naturally occurring active phytochemicals, usually referred to as secondary plant metabolites, had been attributed to have different biological properties which provide protection against diverse diseases and give health benefits to humans and animals (Aathira *et al.*, 2021). The concept of polyherbal formulation (PHF) is well registered in the ancient literature, which has better and extended therapeutic potential as compared to the single herb. *Achyranthes aspera, Catharanthus roseus* and *Momordica charantia* have been individually studied and reported to have significant antihyperglycemic activities and are rich reservoir of pharmacologically established antidiabetic phytoconstituents (Akhtar and Iqbal, 1991; Chattopadhyay, 1994; Sarkar *et al*., 1996). Study with mixture containing all these three herbs have not been carried out in diabetic rat model. Thus, these three plants have been selected to prepare PHF to evaluate the effect on blood glucose level, lipid profile, anti-oxidant activity and other pathological changes in streptozotocin-induced diabetic rat model.

Materials and methods

Plant materials and preparation of extracts

The aerial parts of *A. aspera* were procured from road side area of Ranchi Veterinary College, Ranchi. *Catharanthus roseus* collected from horticulture, BAU and *M. charantia* (MC) were grown from seeds and aerial part of plants were handpicked after flowering and fruiting. The plant materials were identified and authenticated by Central National Herbarium, Botanical survey of India, Howrah, West Bengal with reference number CNH/Tech.11/2021/27. All the plant materials (1 kg) were air dried and separately coarsely powdered in a mixer-grinder. A weight of 200 g of each powdered plant was placed in separate conical flasks and 500 ml of methanol was added and plugged with cotton and placed on magnetic stirrer for 72 h for extraction. After 72 h the supernatant was collected by filtration using Whatman paper No.1 and the solvent was evaporated at 50 ^{o}C in oven to make the crude extract. The residues obtained were stored in airtight bottles in a refrigerator for further use.

Chemicals and reagents

Streptozotocin was obtained from Himedia Private Ltd., Mumbai, India. Kits for biochemical parameters were purchased from ERBA Diagnostic Mannheim GmbH, Germany. All other chemicals and reagents were procured from Himedia Private Ltd.

Development of polyherbal formulation

The PHF was developed by combining the dried extracts of the plant materials based on the LD_{50} of individual plant extracts and oral glucose tolerance test in normal rats. The polyherbal formulation (PHF) were made by mixing *A. aspera*, *C. roseus*, and *M. charantia* extracts in the ratio of 3:1:1 w/w/w respectively in 0.5 per cent of gum acacia dissolved in distilled water as vehicle.

Effect of plant extracts on oral glucose tolerance test

Overnight fasted *Sprague-Dawley* normal rats of 6-8 weeks old of either sex

weighing 130-180 g were divided into five groups having 5 animals in each group. The rats were housed in polypropylene cages with free access to fresh water in departmental animal house at a temperature of 22 ± 2 ºC. Group I was kept as normal control; Group II received standard drug Glibenclamide at 2.5 mg/kg body weight; Group III, IV and V received 150 mg / kg body weight of *A. aspera* extract, *C. roseus* extract, and *M. charantia* extract respectively. The single dose of each extract dissolved in 0.5 per cent of gum acacia was administered orally in the rats. All animals received glucose (2g/kg) orally, 30 min after drug and extract treatment. Blood glucose levels were estimated by tail tipping method using Accuchek-Active Glucometer at 0, 30, 60, 90 and 120 min after treatment.

Preparation of high-fat diet.

The High-Fat Diet (HFD) was prepared by using normal pellet diet, raw cholesterol, and mixture of vanaspati ghee and coconut oil (2:1). Normal rat pellet diet was powdered by grinding and mixed with 2.5% cholesterol and mixture of vanaspati ghee and coconut oil (5%). The mixture was made into pellet form and put into freezer to solidify (Nekha *et al*., 2020).

Diabetic model

The HFD was fed orally to rats for 3 weeks to induce metabolic syndrome. The diabetes was induced in overnight fasted rats by a single intra peritoneal injection of a freshly prepared solution of streptozotocin (45 mg/ kg body weight) in 0.09 M cold citrate buffer having a pH of 4.8 (Tikkanen *et al.*, 1998). The rats were then kept for the next 24 h on 5 per cent glucose solution bottles in their cages to prevent hypoglycaemia and were monitored by periodic estimation of body weight and biochemical testing of fasting serum glucose. Only those rats with persistent blood glucose levels ≥200mg/dl for 7 days after streptozotocin administration were considered diabetic and included in the study.

Grouping and treatment schedule for hypoglycaemic activity

Based on the results of OGTT, we proceeded with 60 day hypoglycaemic activity study. The protocol of the experiment was approved by the Institutional Animal Ethics Committee, Ranchi University. Fifty Sprague-Dawley rats were divided into five groups, each containing ten animals, as follows:

Group I: Normal Control

Group II: Diabetic Control

Group III: Diabetic animals treated with glibenclamide @ dose of 2.5 mg/kg b.wt.

Treatment group 1 (T-1): Diabetic animals treated with PHF @ dose of 375 mg/kg b.wt.

Treatment group 2 (T-2): Diabetic animals treated with PHF @ dose of 750 mg/kg b.wt.

All the treatments were given once a day orally for 60 days on fixed time. On day 30th and 60thof the study, the blood samples were withdrawn from all the experimental animals through retro-orbital plexus puncture in plain and EDTA tubes for biochemical analysis. Finally, five animals from each group were sacrificed by using overdose of carbon dioxide and chloroform on day 30 and the remaining rats on day 60 of the experiment; liver tissues were excised and used for biochemical and oxidative stress analysis.

Haematological parameters

Blood glucose levels were analysed using Accuchek-Active Glucometer (Roche Diagnostic GmbH Mannheim, Germany).

Estimation of lipid profile and biochemical parameters

Lipid profile parameters like total cholesterol (TC), HDL- cholesterol, LDL-cholesterol, triglyceride (TG) and biochemical parameters like alanine aminotransferase (ALT), aspartate amino transferase (AST) were estimated by using standard kits (Erba, Diagnostic Mannheim GmbH, Germany) with automatic biochemistry analyser (CHEM-5 Plus $_{v2}$,Transasia Bio-Medicals Ltd., Solan, HP, India).

Oxidative stress related parameters

Lipid peroxidation (LPO) in tissue

homogenate was estimated in terms of malondialdehyde (MDA) production by the modified method of Stock and Dormandy (1971) as described by Jain (1988).

Superoxide dismutase (SOD) was estimated as per the method described by Madesh and Balasubramanium (1998).

Statistical analysis Data were statistically evaluated by two- way ANOVA using the Graphpad Prism v 4.03 software program (San DIEGO, CA, USA) and the differences were considered statistically significant at *P<0.05 or lower (Snedecor and Cochran,1989).

Results and discussion

Effect of extracts on blood glucose in oral glucose tolerance test

The yield percentage of methanolic extracts of *A. aspera*, *C. roseus* and *M. charantia* were 12.13, 11.00 and 15.23 per cent, respectively.

The hypoglycaemic potential of each herbal extracts was tested by oral glucose tolerance test in normal rats. In OGTT model, it was observed that, at 30 min after starting the glucose tolerance test, the blood glucose level increased rapidly in the normal control groups but in treated groups the glucose induced hyperglycemia was prevented as compared to control group (Fig.1). The glibenclamide, *Catharanthus* and *Momordica* extract significantly reduced the elevation in blood glucose level at 30 min compared to the normal control group (P<0.001).The *Achyranthes* extract also reduced the elevated glucose level at 30 min but the reduction was not significant. Glibenclamide treated group also prevented the glucose induced hyperglycaemia at 30 min and 60 min as compared to normal control (P<0.001). There was not any significant decline of the blood glucose level in the normal control group during the observation period.

Symptoms and bodyweight

Rats of diabetic control group showed symptoms like dullness, sluggish movement, weight loss, polyuria, polydipsia and polyphagia whereas these symptoms were moderate in all other treatment groups except normal control group. The mean body weight in diabetic rats were reduced significantly (p<0.05) at the end of 30th and 60th day of experimental period (Table 1). The results obtained are in agreement with findings of Kumar *et al*. (2008) and Cheng *et al*. (2013) that increased appetite, weight loss and muscle weakness in diabetic rats might be due to insulin deficiency causing protein and fats catabolism due to negative energy balance. Polydipsia may be due to hyperglycaemia, causing intracellular water depletion and thus activating the thirst centres in the brain. PHF treatment groups (T1 & T2) and glibenclamide (G-III) treated group showed significant improvement in body weight, which indicates that polyherbal formulation and glibenclamide prevent the hyperglycemia induced muscle wastage. The results obtained are in agreement with findings of Vijayaraj and Kumaran (2018), Fernandes *et al*. (2007) and Hikmah *et al*. (2015) that the administration of *A. aspera*, *M. charantia* and *C. roseus* herbal extract respectively showed an improvement in mean body weight of diabetic rats. The increased body weight in PHF treated group could be attributed to the better utilization of nutrients, glucose, amino acids, fatty acids and other macro-molecular components due to improved insulin secretion by the beta-cells (Chander *et al*. 2015).

Serum glucose level

Diabetic control animals showed severe hyperglycemia compared to normal rats. It was observed that the standard drug, glibenclamide lowered the blood glucose level significantly, bringing it back to near normal level, whereas the PHF at 375 mg/kg and 750 mg/kg significantly (P < 0.001) decreased the fasting serum glucose level in the diabetic rats on 30th and 60th days, as compared to the diabetic control group (Table 2). The reduction in glucose levels may be due to increase in plasma insulin levels or enhanced transport of blood glucose in the peripheral tissue (Wilcox, 2005).The antihyperglycaemic activity of PHF may be due individual herb's active principles in the polyherbal formulation, stimulating remnant β-cells to release more insulin or improving

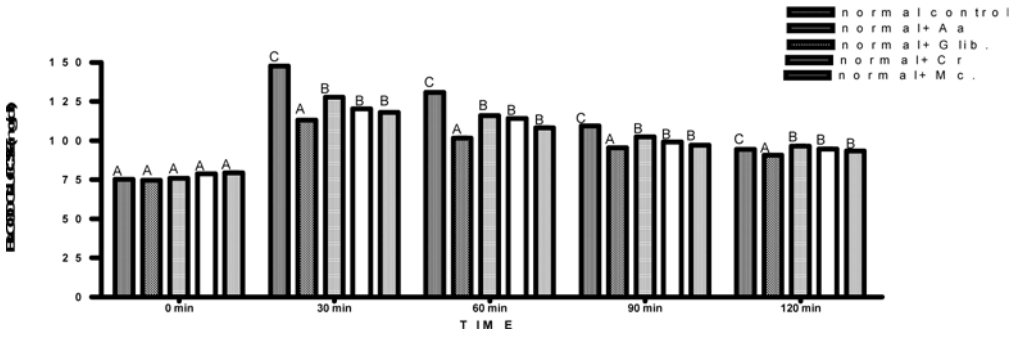

Fig.1. Histogram showing Glucose levels (mg/dl) in rats at different time intervals in different treatment groups under oral glucose tolerance test (OGTT).

insulin action at cellular level. The results were in agreement with Vijayaraj and Kumaran (2018) in *A. aspera* extract; Chattopadhyay, (1991) in *C. roseus*; Sathishsekar and Subramanian (2005) and Sarkar *et al*. (1996) in *M. charantia.* Chattopadhyay, (1994) reported a plant derived natural phytoconstituent named Vinculin, isolated from *C. roseus* having hypoglycaemicactivity. Ng *et al*. (1986) and Raman and Lau (1996), reported that *M. charantia* extract also contains phytoconstituents such as charantin, insulin-like peptides, lecithin and alkaloids which were responsible for hypoglycaemic property. Betaine, achyranthine and β-ecdysones (Akhtar and Iqbal, 1991) were reported to be isolated

Table1. Body weight (g) of experimental animals of different groups.

Body weight	Day 0 (Mean ± SE)	Day30 (Mean ± SE)	Day 60 (Mean ± SE)
Group-I	174.88 ± 3.83^{Aa}	199.47 ± 1.23^{Cb}	248.56 ± 5.7^{Ec}
Group-II	186.86 ± 1.43^{Bc}	147.08 ± 2.62^{Ab}	139.93 ± 5.21^{Aa}
Group-III	193.75 ± 0.56^{Ca}	210.99 ± 1.31^{Db}	221.13 ± 1.12^{Dc}
T-1	172.8 ± 2.51^{Aa}	183.40 ± 0.69^{Bb}	190.9 ± 0.55^{Bc}
T-2	171.12 ± 0.38^{Aa}	188.13 ± 0.38^{Bb}	197.18 ± 4.44^{Cc}

Values with different superscript in a column and row were significantly different (p<0.05).
(Capital superscript- Within column, Small superscript- Within row)

Table 2. Blood glucose (mg/dl) levels in different treatment groups.

Blood glucose	Day 0 (Mean ± SE)	Day30 (Mean ± SE)	Day 60 (Mean ± SE)
Group-I	95.28 ± 0.59^{Aa}	99.29 ± 1.88^{Aa}	98.42 ± 2.61^{Aa}
Group-II	212.83 ± 1.39^{Ca}	262.42 ± 1.35^{Eb}	291.08 ± 1.83^{Dc}
Group-III	204.55 ± 2.51^{Bc}	153.57 ± 3.64^{Bb}	130.26 ± 3.43^{Ca}
T-1	247.95 ± 1.31^{Dc}	190.96 ± 0.29^{Db}	134.45 ± 3.05^{Ca}
T-2	245.56 ± 2.86^{Dc}	169.09 ± 0.53^{Cb}	112.41 ± 3.11^{Ba}

Values with different superscript in a column and row were significantly different (p<0.05).
(Capital superscript- Within column, Small superscript- Within row)

Table 3. The mean ± SE haemoglobin (g/dl) values in different treatment groups.

Groups (Hb%)	Day 0 (Mean ± SE)	Day30(Mean ± SE)	Day 60 (Mean ± SE)
Group-I	11.60 ± 0.02^{Ca}	13.33 ± 0.03^{Cb}	13.9 ± 0.01^{Db}
Group-II	9.75 ± 0.12^{Ab}	9.01 ± 0.12^{Ab}	7.76 ± 0.07^{Aa}
Group-III	10.36 ± 0.1^{Ba}	10.88 ± 0.03^{Ba}	11.76 ± 0.12^{Bb}
T-1	9.84 ± 0.15^{Aa}	11.17 ± 0.09^{Bb}	12.54 ± 0.18^{Cb}
T-2	9.69 ± 0.3^{Aa}	11.79 ± 0.05^{Bb}	14.67 ± 0.14^{Dc}

Values with different superscript in a column and row were significantly different (p<0.05).
(Capital superscript- Within column, Small superscript- Within row)

Table 4. The mean ± SE ALT (IU/L) values in different treatment groups.

Groups (ALT)	Day30 (Mean ± SE)	Day 60 (Mean ± SE)
Group-I	22.80 ± 0.75^{Aa}	23.07 ± 0.42^{Aa}
Group-II	45.97 ± 0.47^{Ea}	55.35 ± 0.34^{Db}
Group-III	34.1 ± 0.37^{Db}	31.93 ± 0.34^{Ca}
T-1	31.72 ± 0.2^{Cb}	28.13 ± 0.1^{Ba}
T-2	29.62 ± 0.41^{Bb}	24.04 ± 0.51^{Aa}

Values with different superscript in a column and row were significantly different (p<0.05).

(Capital superscript- Within column, Small superscript- Within row)

Table 5. The mean ± SE AST (IU/L) values in different treatment groups.

Groups (AST)	Day30 (Mean ± SE)	Day 60 (Mean ± SE)
Group-I	87.35 ± 1.38^{Aa}	87.87 ± 0.92^{Aa}
Group-II	147.66 ± 0.76^{Ea}	170.22 ± 2.27^{Eb}
Group-III	128.50 ± 0.33^{Da}	134.05 ± 0.88^{Db}
T-1	108.91 ± 0.33^{Cb}	101.97 ± 0.49^{Ca}
T-2	102.48 ± 0.54^{Bb}	98.06 ± 0.46^{Ba}

Values with different superscript in a column and row were significantly different (p<0.05).

Table 6. The mean ± SE serum cholesterol (mg/dl) values in different treatment groups.

Groups (TC)	Day30 (Mean ± SE)	Day 60 (Mean ± SE)
Group-I	101.90 ± 1.59^{Aa}	111.32 ± 1.19^{Ab}
Group-II	178.68 ± 0.42^{Ca}	232.84 ± 0.9^{Eb}
Group-III	140.59 ± 0.70^{Bb}	134.21 ± 0.60^{Ca}
T-1	142.38 ± 1.38^{Bb}	138.09 ± 0.47^{Da}
T-2	140.16 ± 0.73^{Bb}	127.45 ± 0.53^{Ba}

Values with different superscript in a column and row were significantly different (p<0.05).
(Capital superscript- Within column, Small superscript- Within row)

from *Achyranthes* which helps in regulating carbohydrate digestion and absorption and thus responsible for hypoglycaemic activity.

Haemoglobin (Hb %)

The mean Hb% in diabetic control rats were observed to decline drastically from 30th to 60th day of experiment (Table 3). Decreased Hb content in diabetic rats might be due to increased formation of glycosylated Hb. In agreement with the present results, Emmanuel *et al*., (2010)also reported that in *diabetes* ailment the excess of glucose present in the blood reacts with haemoglobin to form glycated hemoglobin (HbA_{1C}) thus exhibiting low level of Hb in diabetic animals. The improvement in Hb values in PHF (T-1,T-2) treated groups might be due to reduction in blood glucose levels thereby decreasing the excess glucose reacting with haemoglobin forming glycosylated haemoglobin or might be due to improvement in insulin secretion by β-cells; thus reducing the severity of hyperglycaemia; were reported by Vijayaraj and Kumaran (2018), Jayanthi *et al*. (2010) and Ali *et al*. (1993) in *A. aspera, C. roseus* and *M. charantia* extracts treated diabetic animals, respectively.

Assessment of liver toxicity enzyme test

Serum ALT and AST mean values in diabetic rats were significantly (P<0.001) higher as compared to normal control animals throughout the study period (Table 4, 5), similar findings were also reported by many workers (Shibib *et al*., 1993; Chaudhari *et al*., 2009). Streptozotocin induced hyperglycaemia causes elevation of plasma levels of ALT and AST, which are significant markers of liver (hepatic) abnormality. Supporting our findings, Ohaeri (2001) has reported that the structure of liver was modified in diabetic rats. Therefore, elevated activities of ALT and AST in serum

Table 7. The mean±SE serum triglycerides (mg/dl) values in different treatment groups.

Groups (TG)	Day30 (Mean ± SE)	Day 60 (Mean ± SE)
Group-I	84.29 ± 0.45^{Aa}	85.17 ± 0.60^{Aa}
Group-II	196.02 ± 2.02^{Ea}	220.52 ± 1.55^{Eb}
Group-III	140.04 ± 1.94^{Db}	128.66 ± 0.35^{Da}
T-1	132.13 ± 0.94^{Cb}	123.97 ± 0.66^{Ca}
T-2	121.03 ± 1.08^{Bb}	108.46 ± 1.24^{Ba}

Values with different superscript in a column and row were significantly different (p<0.05).

Table 8. The mean ± SE serum LDL-cholesterol (mg/dl) values in different treatment groups.

Groups (LDL-c)	Day30 (Mean ± SE)	Day 60 (Mean ± SE)
Group-I	52.68 ± 0.32^{Aa}	54.89 ± 0.59^{Aa}
Group-II	151.78 ± 0.28^{Ea}	171.12 ± 0.45^{Eb}
Group-III	85.03 ± 0.27^{Db}	78.19 ± 0.26^{Da}
T-1	72.88 ± 0.33^{Cb}	67.33 ± 0.23^{Ca}
T-2	68.74 ± 0.23^{Bb}	62.24 ± 0.32^{Ba}

Values with different superscript in a column and row were significantly different (p<0.05).
(Capital superscript- Within column, Small superscript- Within row)

Table 9. The mean±SE serum HDL-cholesterol (mg/dl) values in different treatment groups.

Groups (HDL-c)	Day30 (Mean ± SE)	Day 60 (Mean ± SE)
Group-I	30.32 ± 0.36^{Ca}	34.54 ± 0.27^{Cb}
Group-II	17.88 ± 0.55^{Ab}	15.41 ± 0.32^{Aa}
Group-III	25.73 ± 0.35^{Ba}	29.4 ± 0.23^{Bb}
T-1	37.69 ± 0.20^{Da}	40.83 ± 0.32^{Db}
T-2	38.87 ± 0.25^{Ea}	42.02 ± 0.05^{Eb}

Values with different superscript in a column and row were significantly different (p<0.05).

Table 10. The mean ± SE SOD activity(U/mg protein) in liver tissue collected from rats under different treatment groups.

Groups (SOD)	Day30 (Mean ± SE)	Day 60 (Mean ± SE)
Group-I	25.43 ± 3.78^{Da}	26.72 ± 4.0^{Ca}
Group-II	19.49 ± 2.66^{Ab}	16.45 ± 2.12^{Aa}
Group-III	21.53 ± 3.03^{Ba}	21.85 ± 6.95^{Ba}
T-1	23.46 ± 3.4^{Ca}	26.98 ± 4.02^{Cb}
T-2	25.7 ± 3.79^{Da}	29.78 ± 4.54^{Db}

Values with different superscript in a column and row were significantly different (p<0.05).
(Capital superscript- Within column, Small superscript- Within row)

might be due to the drop out of these enzymes into the blood stream from the liver cytosol due to increased hepatocytes membrane permeability or membrane damage (Concepcion *et al*., 1993); or due to increased activity of amino acid, responsible for elevated gluconeogenesis and ketogenesis (Nikkila and Kekki, 1973). In the present study, PHF and glibenclamide treated groups showed significant (P<0.001) reduction in ALT and AST levels as compared to diabetic control animals. This result may be due to the ameliorative effect of the individual herbs present in the polyherbal formulation (Shah *et al*., 2010). Findings are in agreement with previous reports by Bafna and Mishra (2004), Al-Shaqha*et al*. (2015) and Dandagi *et al*. (2008) related to hepatoprotective activity of *A. aspera, C. roseus* and *M. charantia* extracts, respectively in restoring the elevated levels of ALT and AST in diabetic rats. Chaudhari *et al*. (2009) also attributed the hepatoprotective effect of *M. charantia* due to presence of flavanoids, ascorbic acid and other components such as saponins, tannins, triterpenes and alkaloids in the extract etc.

Table 11. The mean ± SE MDA level (nmol/mg protein) in liver tissue collected from rats under different treatment groups.

Groups (MDA)	Day30 (Mean ± SE)	Day 60 (Mean ± SE)
Group-I	29.19 ± 0.36^{Ca}	29.65 ± 0.41^{Ca}
Group-II	43.10 ± 0.63^{Ea}	50.998 ± 0.38^{Eb}
Group-III	33.96 ± 0.67^{Da}	31.46 ± 0.41^{Da}
T-1	21.80 ± 0.41^{Bb}	17.81 ± 0.14^{Ba}
T-2	19.00 ± 0.24^{Ab}	15.04 ± 0.26^{Aa}

Values with different superscript in a column and row were significantly different (p<0.05).
(Capital superscript- Within column, Small superscript- Within row)

Lipid profile

In *diabetes mellitus*, hyperglycemia is accompanied with dyslipidemia that is characterized by increase in total cholesterol, triglyceride, LDL cholesterol and decrease in HDL cholesterol (Table 6, 7, 8 and 9). The abnormally high concentration of serum lipids in diabetics is mainly due to increased congregation of free fatty acids from the peripheral fat depots (Gupta *et al*., 2009). In the present study, the mean values of HDL-cholesterol were significantly (P<0.001) increased whereas TC, TG and LDL-cholesterol were significantly (P<0.001) decreased in both PHF and glibenclamide treated groups as compared to diabetic control on day 30 and 60. Supporting to our findings, Jayanthi *et al*. (2010), reported a significant lowering of serum total lipid levels treated with *C. roseus* in streptozotocin induced diabetic rats. Significant improvement in hyperlipidemia were in agreement with the findings of Khanna *et al*. (1992), who showed alcoholic extract of *A. aspera* had lowered serum cholesterol in triton induced hyperlipidemic rats. The findings were also in agreement with Krishnakumari and Priya (2006), who reported hypolipidemic efficacy of *A*. *aspera* in sesame oil fed rats. In agreement with present results, Nerurkar (2005) also reported that treatment with methanolic extract of *M. charantia* significantly decreased the total serum cholesterol, triglycerides, low density lipoproteins and increased the high-density lipoproteins in obese rats. This might be due improved insulin release, enhanced uptake of glucose and better utilization of nutrients like glucose, amino acids, fatty acids and other macromolecular components (Singh *et al.,* 1989).

Antioxidant defense system

Superoxide dismutase (SOD) is an antioxidant enzyme that catalyses the dismutation of superoxide to produce hydrogen peroxide and oxygen. Increased production of superoxide ion leads to elevation in SOD activity to alleviate the auto-oxidation and oxidative stress and thus provides cellular protection against the damage caused by free radicals and ROS (Kant *et al*., 2011). Increased levels of lipid peroxides and reactive oxygen species by streptozotocin cause alkylation, breakage of DNA strands or tissue damage through peroxidation of unsaturated fatty acids finally leading to death of beta cells (Alireza *et al*., 2009). The PHF treated animals showed improved oxidative stress (Table 10 and 11), which may be due to the free radical scavenging properties of the individual herbs present in it or due to prevention of glucose auto-oxidation, protein glycation and the polyol pathway that generates free radicals (Atalay and Laaksonen, 2002). Results related to improved oxidative stress markers in diabetic animals treated with PHF (T-1, T-2) in the present study are in agreement with previous findings reported by Vijayaraj and Kumaran (2018), Salah *et al*. (1995), and Sathishsekar and Subramanian (2005) related to antioxidant activity of *A. aspera, C. roseus* and *M. charantia,* respectively.

Conclusion

Polyherbal formulation (3:1:1w/w/w/) with extracts of aerial plant parts of *A. aspera, C. roseus* and *M. charantia* respectively at 375 and 750 mg/kg doses, showed ameliorative effect against streptozotocin

induced alterations in diabetic rats, due to active principles. These findings suggested the hypoglycemic, hypolipidemic, hepatoprotective and antioxidant potentials of the PHF and thus help in preventing future complications of diabetes.

Conflict of interest

We declare that we have no conflict of interest.

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Association of Butyrophilin gene polymorphism (A465G) with milk production traits in Holstein Friesian crossbred cattle of Kerala

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Abstract

The butyrophilin (BTN1A1) gene is found at a quantitative trait locus (QTL) in cattle for milk production traits. The effect of a single nucleotide polymorphism (SNP) A465G in exon 8, which causes a lysine to arginine transition, on milk production attributes in Holstein Friesian crossbred cattle from Kerala was investigated in this study (151 numbers). Using custom synthesised primers, a 90-bp fragment encompassing the polymorphic region was amplified from genomic DNA isolated. Genotyping was carried out by high resolution melt curve analysis (HRM) and two genotypes KK (0.25) and KL (0.75) were detected based on melting temperature and melt curve patterns. Sanger's sequencing and sequence analysis of representative samples confirmed the genotypes. Chi-square test showed that the population was not distributed as per Hardy-Weinberg equilibrium (p≤0.05). The relationship between the A465G transition and milk production traits like 305 day milk, fat, SNF yields, fat and SNF per cent was determined by general linear model-analysis of variance (GLM-ANOVA). In the model herd, season of calving, parity of animal (non-genetic factors), and genotype were considered fixed variables and milk production traits as dependent variable. The study revealed significantly higher (p≤0.01) milk fat and SNF per cent for KK genotype (4.14±0.08 %; 7.86±0.06 %) than KL genotype (3.91±0.07 %; 7.73±0.05 %). The butyrophilin gene polymorphism (A465G) can be recommended as a marker for higher milk fat and SNF per cent in future breeding programmes in crossbred cattle of Kerala.

Keywords: *Butyrophilin, SNP, high resolution melt curve, crossbred cattle, milk production traits*

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Butyrophilin (*BTN1A1*) gene is mapped to bovine autosome 23 (Taylor *et al*., 1996) and in the same region quantitative trait loci (QTL) for milk production traits (Ashwell *et al.*, 1996; Bennewitz *et al.*, 2004). Previous researchers suggested *BTN1A1* as a candidate gene for milk production (Komisarek *et al.*, 2006; Wenjun *et al*., 2009; Muszynska *et al*., 2010; Al-Waith 2019) and disease resistance traits (Smith *et al*., 2010) in cattle. Butyrophilin gene is present on juxta-telomeric region of bovine leucocytic antigen (BoLA) complex (Ashwell *et al*., 1996) and span over 7003 bp length with eight exons and seven introns (Vishwanath, 2014). Butyrophilins are type I trans membrane proteins belonging to immunoglobulin (Ig) super family with an extra cellular andcytoplasmic domain. Cytoplasmic domain of *BTN1A1* tightly binds to xanthine dehydrogenase (*XDH*) to form a supra-molecular complex. This complex binds to adipophilin in the phospholipid bilayer of milk secretory granule and pinches off the mammary gland epithelial cell in zipper like fashion (Robenek *et al*., 2006).

Many molecular markers in candidate genes for milk production such as *Diacylglycerol-o-transferase 1* (Lali and Anilkumar, 2016), *Beta 1,4-galactosyltransferase-1* (Valsalan *et al.,*2021), *Leptin* (Lali and Bindu, 2015) and *Osteopontin* (Lali *et al.,* 2020) were studied in crossbred cattle of Kerala. It is worth to note that, majority of SNPs act as indirect markers and the influence on production traits depends on state of linkage disequilibrium (LD) in cattle. Schmid and Bennewitz (2017) explained that the LD between the marker and the QTL might be affected by the genetic recombination during gamete formation. It reiterates the importance of association results of local populations to confirm the effects of markers especially indirect markers. The influence of *BTN1A1*gene polymorphisms was not studied yet in the crossbred cattle population of Kerala. The present study was designed to analyse the effect of polymorphism in*BTN1A1* gene (A465G) on milk production traits in Holstein Friesian crossbred cattle of Kerala.

Materials and methods

Estimation of milk production traits

Milk samples were collected from 151 crossbred cattle maintained at University Livestock Farm and Fodder Research Development Scheme (ULF-FRDS), Mannuthy and cattle breeding farm (CBF), Thumburmuzhy once in a month for ten months. The samples were brought to laboratory under refrigerated conditions. Data regarding animal number, date of calving, parity, test day milk yield, recording month and date were obtained from farm records. Test day fat per cent was estimated from automatic milk analyser (MRC instruments) only after routine standardisation with Gerber's centrifugation method. Data regarding test day SNF per cent was derived from milk analyser by running samples. Test interval method (TIM), according to guidelines of international committee for animal recording (ICAR, 2020) was used to calculate 305 day yields of milk, fat and SNF along with 305 day milk fat and SNF per cent.

High resolution melt curve analysis

A volume of 5 mL blood was collected from jugular vein of 151 crossbred cattle in a EDTA coated vial. Genomic DNA was isolated from blood by standard phenol chloroform extraction method (Sambrook and Russell, 2001). Isolated DNA was checked for concentration and purity by Nanodrop spectrophotometry. Quality was assessed by 0.8 per cent agarose gel electrophoresis.

Polymorphism analysis and genotyping were carried out using High resolution melt curve (HRM) analysis (Desai *et al.*, 2021) in Eco Real-Time PCR system (Illumina). Primers were custom synthesised using primer3 V.0.4.0 software and a gradient PCR was carried out to fix optimum annealing temperature and specific amplification was detected by running samples on 2.5 per cent agarose gel using molecular weight marker of 50 bp size. The HRM reaction was carried out using SSO FAST EVA GREEN super mix, forward (**5**´GCCCTTCTTCTGCTTGTGGT**3**´) and reverse (**5**´TCAGCAACTACCATGACTCCC**3**´) primers and template DNA. Thermal profile of reactions include 94°C for 5 min, 94°C for 30s, 62.5°C for 30s, 72°C for 30s followed by melt curve analysis. The results were confirmed

by Sanger's sequencing the representative samples from each genotype after detecting them using melting temperature (T_m) shift and melt curve pattern. Further, the genotype and allele frequencies were calculated and population was checked for Hardy Weinberg equilibrium using *chi*-square test.

Association with milk production traits

In order to study the association of A465G polymorphism with milk production traits, General linear model-Analysis of variance (GLM-ANOVA) was performed using SPSS version 24.0. Non-genetic factors such as herd, season of calving (October to January – post monsoon, February to May – summer, June to September – monsoon) and parity (1 to 4) and milk production traits 305-day milk yield, fat yield, fat per cent, SNF yield and SNF per cent as dependent variables.

The model was $Y_{ijklm}= \mu+H_i+S_j+P_K+G_l+e_{ijklm}$

Where,Y_{ijklm} – trait of m^{th} cow in i^{th} herd, j^{th} season, k^{th} parity and belonging to l^{th} genotype, μ - population mean of trait, H_i – effect of i^{th} herd (i = 1 or 2), S_j – effect of j^{th} season (j = 1 to 3), P_k – effect of k^{th} parity (k = 1 to 4), G_l – effect of l^{th} genotype (l = 1 or 2) and e_{ijklm} – Random error.

Results and discussion

A single nucleotide polymorphism in the exon 8 of *BTN1A1*gene resulting from adenine to guanine transition was studied in detail in the present study by high resolution melt curve analysis in HF crossbred cattle of Kerala.

High resolution melt curve analysis

Gradient PCR detected optimum annealing temperature of 62.5 °C. Specific amplification was confirmed by running amplicons through 2.5 per cent agarose gel (Fig. 1). Melt curve analysis clearly depicts presence of two curve patterns representing two genotypes KK and KL (Fig. 3) in the studied population. According to classification of Venter *et al.* (2001), the typical melting temperature shift of C/T or G/A mutations should be >0.5°C. Similarly, the genotypes of HRM analysis of the current study showed a melting temperature shift around 0.6°C. The chromatograms of both genotypes are depicted in Fig. 3. The sequence results were checked for any other genetic variations in the population since, multiple variations in the same amplicon will interfere the interpretation of HRM results.

The allele K (0.63 %) and genotype KL (0.75 %) were found to be frequent in the studied population (Table 1). The frequency of dominant allele K in different studies were0.88 by Komisarek and Dorynek (2003), 0.83 by Bhattacharya *et al*. (2006), 0.86 by Sadr *et al*. (2008), 0.88 by Rengarajan (2011), 0.89 by Vishwanath (2014) and 0.86 by Al-Waith (2019). Thus, the results of K

Table 1. Genotype and allele frequencies for SNP A465G in bovine *BTN1A1* gene

SNP	Genotype frequencies			Allele frequencies		*Chi*-square value
A465G (n=151)	KK/0.25 (37)	KL/0.75 (114)	LL/0 (0)	K/0.63	L/0.37	55.54^S

S-Significant (p≤0.05)

Table 2. Effect of SNP A465G on milk production traits in crossbred cattle of Kerala

Sl. No.	Trait (Mean ± SE)	A465G		p- value
		KK	KL	
1.	305 day milk yield (kg)	2764.35±157.96	2850.68±128.20	p>0.05
2.	Fat yield (kg)	112.40±5.50	109.73±4.52	p>0.05
3.	Fat per cent	4.14±0.08	3.91±0.07	p≤0.01
4.	SNF yield (kg)	216.05±11.75	219.70±9.54	p>0.05
5.	SNF per cent	7.86±0.06	7.73±0.05	p≤0.01

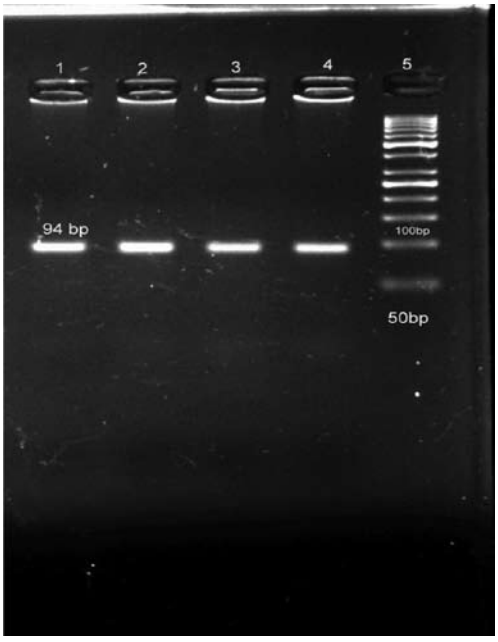

Fig.1. *BTN1A1* gene fragment (94 bp) on 2.5 % agarose gel
Lane 1-4: 94 bp product enclosing exon 3
Lane 5 : 50 bp ladder

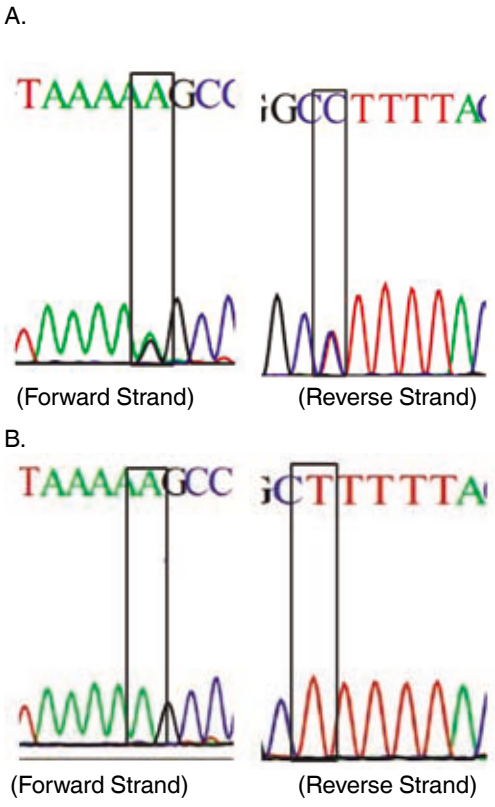

Fig. 2. Chromatogram depicting SNP A465G in exon eight of *BTN1A1* gene in crossbred cattle of Kerala. A and B – Chromatogram depicting KL and KK genotypes, respectively

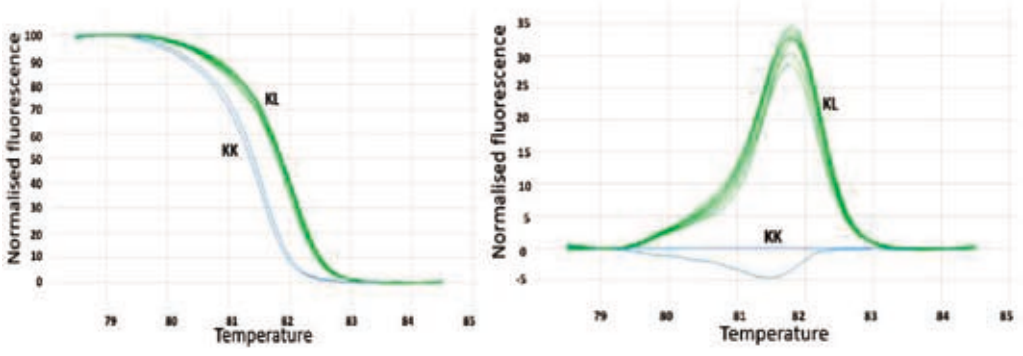

Fig. 3. Normalised and difference melt curve of polymorphism A465G representing two different curve patterns for genotypes KK (blue) and KL (green), respectively.

allele dominance was in accordance with all aforementioned studies. The genotype LL was not detected in the present study which was in agreement with Sadr *et al*. (2008), Rychtarova *et al*. (2014), Vishwanath (2014) and Al-Waith (2019). *Chi*-square test showed that the population was not in Hardy-Weinberg equilibrium ($p \leq 0.05$). Natural selection may be going on favouring the K allele in crossbred cattle population. However, the status of HW equilibrium has to be further examined in a larger population.

Association with milk production traits

The two genotypes KK and KL of A465G transition of HF crossbreds of Kerala differed significantly ($p \leq 0.01$) with respect to fat and SNF per cent (Table 2) as per GLM ANOVA considering herd, season of calving, parity of animal (non-genetic factors) and genotype as fixed variables and milk production traits as dependent variables. Similar results were obtained by Rengarajan (2011) in milk fat per cent and current findings were also in accordance with Vishwanath (2014). However, contradictory to the present results, animals with KK genotype in a study by Komisarek *et al*. (2006) showed significant higher yields of milk, fat and SNF. Furthermore, Rychtarova *et al*. (2014) detected no association of the SNP A465G with milk production traits and it agrees with the current results except for fat and SNF per cent. Al-Waith (2019) identified significant difference between two genotypes KK and KL with respect to milk yield (KK had higher milk yield) and fat per cent (KL had higher milk fat per cent). Inconsistent association analysis results could be the result of population substructure creation, null alleles in population or excessive selection pressure (Lali *et al*., 2020).Thus, A465G polymorphism can be suggested as a potential marker for obtaining a good selling price to a farmer for milk as the fat and SNF per cent in milk determines the milk pricing in Kerala.

Conclusion

The study designed HRM analysis to genotype A465G transition in exon eight of bovine *BTN1A1* gene. Genotype KK had significantly higher fat and SNF per cent in HF crossbreds of Kerala. The favourable allele K was found to be frequent in the population and it indicates that selection undergoes towards the favourable genotypes. Presently more emphasis is on milk composition traits and so extensive studies such as genome wide association studies are required to find out the genes regulating the milk components in cattle. Along with these, studies directing to find influence of stage of lactation are also necessary as it was found that this particular factor has effect on milk composition traits (Prasad and Subramanyam, 1986).

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Conflict of interest

The authors report no conflict of interest.

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RESEARCH ARTICLE
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Occurrence of dermatophytosis in dogs from Thrissur, Kerala

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Abstract

Dermatophytosis is one of the most common skin diseases that affect dogs. Geographic factors like temperature and humidity play an important role in determining prevalence of dermatophytosis. The aim of this study was to determine the macroscopic and microscopic identification of different types of dermatophytes from the dogs presented to University Veterinary Hospital, Kokkali and Teaching Veterinary Clinical Complex, Mannuthy. Skin scrapings and hair were collected from the dogs presented with complaint of alopecia and pruritus. Each sample was cultured on Sabouraud Dextrose Agar (SDA). The cultures were incubated at room temperature for maximum of four weeks. The isolates of fungi were examined macroscopically and microscopically. Lactophenol cotton blue staining technique was used for fungi morphology identification. The most common type of dermatophytes affecting dogs in Thrissur district, Kerala were Trichophyton spp. (68 per cent), Microsporum spp. (32 per cent) and other non-dermatophytes fungi viz. Aspergillus spp., Sporothrix spp., and curvularia spp. This study could assist investigators for understanding the prevalence of the dermatophytes and in zoonotic aspects.

Keywords: *Dermatophytosis, Sabouraud Dextrose Agar.*

Dermatophytosis is the most common superficial, infectious and highly contagious mycosis of both animals and humans. Dermatophytosis is caused by a group of fungi known as dermatophytes (Simpanya and Baxter, 1996). The various studies on prevalence of dermatophytosis reported that 49.7 per cent animals suffered with dermatophytosis in different regions of Iran (Shokri and Khosravi, 2016). Prevalence of dermatophytosis was more in dogs and cats (78.7 per cent) than in domestic livestock (33 per cent) in western parts of India (Murmu *et al.,* 2015). In dogs, prevalence of dermatophytosis ranged from four percent to 10 per cent while higher prevalence had been reported in Turkey (Brilhante *et al.,* 2018). Contaminated environment, inanimate objects,

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animals with subclinical or clinical infections, and animals that were mechanical carriers of the spores on their hair coat acted as reservoirs of infection for both people and animals (Moriello *et al.,* 2017). Based on ecological distribution, dermatophytes are divided into three main groups: *geophilic, zoophilic* and *anthropophilic.* Geophilic is a type of dermatophyte present in soil like *Microsporum gypseum.* It is widespread in warm, humid, tropical and subtropical environments. Zoophilic dermatophytes infect animal hosts and are rarely found in soil (Abdalla and Wisal, 2018). Anthropophilic fungi can infect humans and animals, but cannot survive in soil and includes *Trichophyton rubrum* and *Epidermophyton floccosum* (Moriello *et al.,* 2017). Dermatophytic infection is common worldwide with incidence rates increasing gradually because of the increase in rearing of pets. Mycotic lesions were extremely variable but most of them showed scaliness and alopecia (David *et al.,* 2004).

Materials and methods

The present study was conducted in the Department of Veterinary Epidemiology and Preventive Medicine during the period from January 2021 to September 2021. This study mainly aimed to detect the prevalence of dermatophytes in canine population through culture. A total of 100 dogs with the clinical signs of dermatitis like localized or generalized alopecia, pruritus and inflammatory lesions presented to University Veterinary Hospital (UVH), Kokkalai and Teaching Veterinary Clinical Complex (TVCC), Mannuthy were selected for this study. Selection criteria includes: Dogs with superficial skin lesions clinically diagnosed as dermatophyte infection, dogs with complaint of localized and generalized alopecia, pruritus and localized inflammatory lesions. Exclusion criteria includes: dogs whose skin scrapings were negative for fungal spores and dogs with moist skin infection were not screened for dermatophytosis which could be indicative of non-fungal infection in most of the cases.

Collection of samples

The samples such as skin and hair were collected from infected dogs with clinical signs suggestive of dermatophytosis presented to UVH, Kokkalai and TVCC, Mannuthy during the period from January 2021 to September 2021. A total of 100 cases were having signs of hair loss, scaling, crusts and desquamation were examined for dermatophytosis. Age, gender and season wise categorization of isolates were done for future study.

Direct microscopic examination

Skin scrapings from all the 100 dogs which showed either the presence of fungal spores in the hair shaft or presence of fungal hyphae in epithelial cells on KOH preparation were selected for the further isolation and identification.

Cultural examination and identification

All collected skin samples were subjected to cultural examination on Sabouraud dextrose agar with 0.05 per cent chloramphenicol. The samples were incubated at room temperature for two to four weeks to allow the sufficient growth. Characterisation of fungi morphology was carried out by macroscopic identification of fungi colony colour, growth rate, pigmentation and texture. Tease mount method and cellophane tape methods were used for microscopic identification of dermatophytes using Lactophenol Cotton Blue stain

Results and discussion

Among the 100 skin scraping samples, 25 samples showed colony characteristics of dermatophytes on SDA. In the remaining 60 per cent samples, non-dermatophyte fungal growth was identified based on the cultural characteristics on SDA, which included *Aspergillosis* spp., *Trichoderma* spp., *Sporothrix* spp., *Fusarium* spp. and *Curvularia* spp. No growth could be detected in 15 per cent samples. Among the 25 dermatophyte isolates, different types of dermatophyte species could be detected with higher occurrence of *Trichophyton* spp. (68 per cent), *Microsporum* spp. (28 per cent) and *Epidermophyton floccosum* (four per cent). Within the genus Trichophyton, different species could be isolated based on the microscopic appearance of microconidia with higher prevalence of *T. mentagrophyte* (64.7 per cent) and *T. rubrum*

(35.3 per cent). Within the genus Microsporum, *M. gypseum* (71.4 per cent), *M. nanum* (28.6 per cent).

Characterisation of *T. mentagrophytes* was carried out by appearance of flat, white to cream colonies, with a powdery to granular surface. Reverse pigmentation was usually yellow-brown colour. Septate hyphae had conidiophores extended from them. Numerous single-celled microconidia were formed, often in dense clusters. Microconidia were smooth-walled, and were predominantly spherical to subspherical in shape. Sessile (not on stalk) microconidia were produced in rather dense, grape like clusters on conidiophores. Pencil shaped macroconidia with 3 to 8 cells dividing the interior were produced. Colony morphology and appearance on lactophenol cotton blue staining (100x) is shown in Fig. 1. a, b, c and d.

Characterisation of *Trichophyton rubrum* was done by downy to cottony appearance of colonies with fine white aerial mycelium at surface. Surface was white in colour. Colony growth at bottom produce typically wine red to brown colour pigment. Fungi produced septate hyphae and clavate shaped microconidia and few macroconidia. Microconidia appearance was birds-on-wire appearance. Macroconidia were smooth walled and narrow club shaped (Fig. 2. a, b, c and d).

Characterisation of *Epidermophyton floccosum* was done by appearance of mustard yellow or yellowish-brown colour colonies. Colonies were velvety or felty in texture and folded in appearance as growth progressed. It has septate hyphae without microconidia. Macroconidia developed as lateral or terminal out growths from matured hyphae. Macroconidia were thin walled, containing 2to 5 cells singly or in clusters (Fig. 3. a, b, c and d).

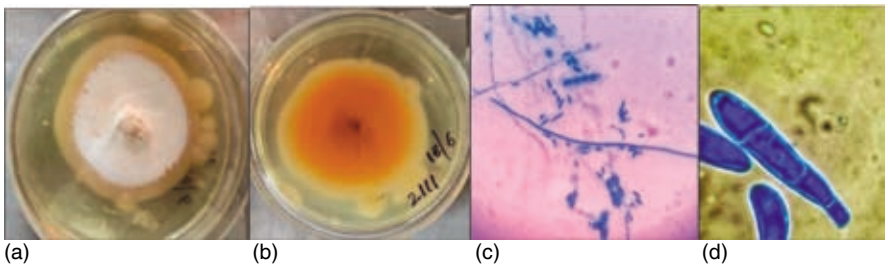
Characterisation of *Microsporum gypseum* was done by appearance of yellowish buff to a dark cream colour colony. Reverse was yellow to orange tan or brownish red in colour. Produced septate hyphae along which sessile or stalked clavate or club shaped microconidia grew. Macroconidia were thin walled, verrucose with bumpy surface and contained about 2 to 6 internal cells (Fig.4. a, b, c and d).

Characterisation of *Microsporum nanum* was done by appearance of thin, powdery and soft fibrous colonies, white at the centre, becoming light yellowish-brown towards the colony margin. In the younger colonies reverse side appears brownish-orange and reddish-brown in older colonies. The macroconidia were ovoid in shape and consisted of not more than three cells. Rare to moderate numbers of microconidia could be found (Fig. 5. a, b, c and d).

According to present epidemiological study, 25 per cent of dermatophyte occurrence was noticed in the Thrissur district of Kerala. Similar percentage of canine dermatophytosis incidence was noticed by Bernardo *et al.,* (1989) 21 per cent, Pinter and Stritof (2004) 24.55 per cent and Sever *et al.* (2017) 29.6 per cent. Some other authors reported lower incidence of dermatophytosis at various regions of world with 14 per cent (Brilhante, *et al.,* 2003), 18.7 per cent (Seker and Dogan, 2011) and 13.5 per cent (Sigirci *et al.,* 2019) in their respective studies, however, some researchers found high level of incidence about 36 per cent (Caretta *et al.,* 1989), 58 per cent (Ranganathan *et al.,* 1998), 45 per cent. (Guzman-Chavez *et al.,* 2000) and 49.5 per cent (Nweze, 2011) in their corresponding studies. The variation in incidence may be due to the differences in various geographical factors like temperature, humidity and rainfall. It also depends on size of the sample, sampling procedure.

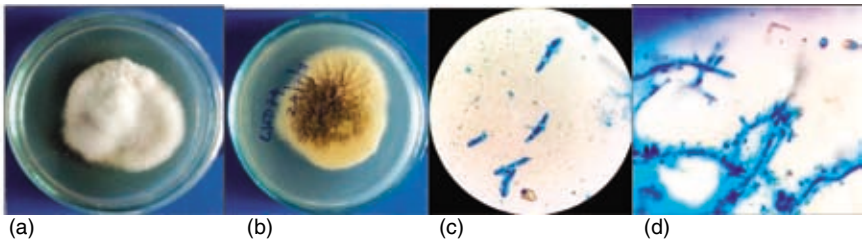
Incidence of dermatophytosis was most commonly seen among puppies of less than six months of age (48 per cent) than animals between six months to two years (36 per cent) and adult dogs from two years to six years of age (16 percent) (Fig.6). The higher incidence of dermatophytosis in puppies might be due to immaturity of the immune system. This is in accordance with Cafarchia *et al.* (2004), Debnath *et al.* (2005), Copetti *et al.* (2006), Seker and Dogan (2011), Cunha *et al.* (2017) and Minnat (2019).

Dermatophytosis occurrence was slightly high in male dogs (52 per cent) than female dogs (48 per cent), which was not significant. Similar findings were noticed by



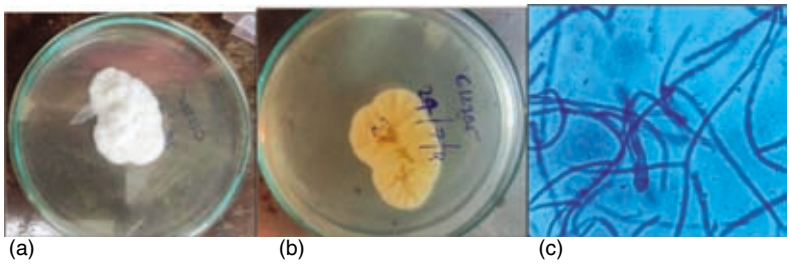
(a) (b) (c) (d)

Fig. 1. *T. mentagrophyte;* ***a.*** Flat, white to cream powdery colony; b. Yellow-brown colour pigment on reverse; c. Single-celled microconidia; d. pencil shaped macro conidia.



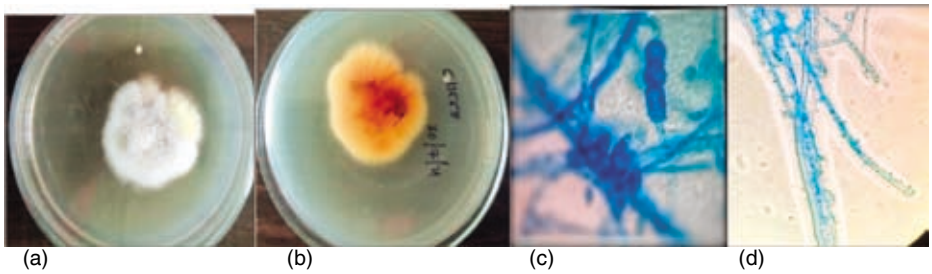
(a) (b) (c) (d)

Fig. 2. *T. rubrum.* a. Downy to cottony (upside); b. Brown in colour (Reverse); c. Smooth walled macroconidia; d. Birds on wire appearance of microconidia.



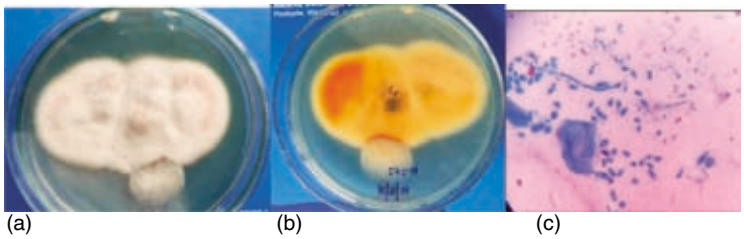
(a) (b) (c)

Fig. 3. *Epidermatophyton floccosum.* a&b. Mustard yellow colonies with folded appearance; b. Four celled macroconidia



(a) (b) (c) (d)

Fig. 4. *Microsporum gypseum* a. Dark cream colour (upside); b. Yellow to orange tan colour (reverse) c. Verrucose, bumpy surface macro conidia; d. Septate hyphae, club shaped and microconidia.



(a) (b) (c)

Fig. 5. *Microsporum nanum* a**.** Powdery and soft fibrous colony; b. Yellowish-brown towards margin (reverse); c. Macroconidia;

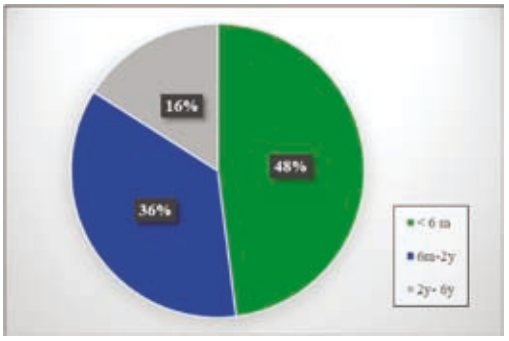


Fig. 6. Age wise distribution of dermatophytosis

Cafarchia *et al.* (2004), Guzman-Chavez *et al*., (2000), Debnath *et al.* (2005) and Seker and Dogan (2011). They explained this might be due to difference in composition of sebum of male dogs when compared with female dogs. Similar findings were noticed by Pinter and Stritof (1999) who reported that incidence was also influenced by nature of the study population in the study.

Conclusion

This study was performed to find out the most common cause of canine dermatophytosis in Thrissur district of Kerala. The results of this study revealed that dermatophyte infections are mostly prevalent in young animals than adults. Dermatophytosis occurrence was higher during summer season than monsoon and winter. *Trichophyton mentagrophyte* was the most common isolate in the study which has the zoonotic importance. So, such dogs can act as potential sources for human infections. This study can assist investigators in understanding the prevalence of dermatophytes in Thrissur district and their zoonotic implication.

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Conflict of interest

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SHORT COMMUNICATION
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A case report on feline idiopathic cystitis#

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Abstract

Feline idiopathic cystitis is similar to human interstitial cystitis as the occurrence of both diseases is characterised by the involvement of stress, severe abdominal pain and a reduction in the protective glycosaminoglycan layer of the bladder. The present case report involves the occurrence of feline idiopathic cystitis in a tomcat. The animal was presented with the signs of anorexia, vomiting, haematuria, dysuria, pollakiuria, periuria and urethral obstruction. Based on the results of haematology, serum biochemistry, imaging techniques, urinalysis and culture results, the case was diagnosed. Urethral patency was re-established by urethral catheterisation and the case was managed with a combination of medical, dietary and multi-model environmental enrichment therapy.

Keywords: *Feline idiopathic cystitis, interstitial cystitis, glycosaminoglycans*

Feline idiopathic cystitis (FIC) is the most common cause of feline lower urinary tract disease and is similar to the human interstitial cystitis (IC), which is characterised by severe painful frequent urination without an identifiable cause (Gunn-Moore, 2003). The term FIC or feline interstitial cystitis was coined by Buffington *et al*. (1996) to describe chronic lower urinary tract signs (LUTS) in cats where an etiology cannot be identified in any of the diagnostic tests. Therefore FIC is always a diagnosis of exclusion (Westropp and Buffington, 2016). As per Gunn-Moore (2003), some cats with FIC had lowered urinary excretion of glycosaminoglycans (GAG) as a result of defective GAG

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layer which is the protective layer of bladder. This exposes the urothelium and it comes directly in contact with the noxious substances in urine, stimulating the sensory neurons and initiating inflammation. Studies have reported increased incidences of FIC in middle aged, stressed, obese, indoor housed cats fed on dry diet. Incidences were also higher in cats using indoor litter box, and in those living in a multi-cat households (Taylor, 2015). The management of FIC involves medical therapy, dietary therapy and multi-model environmental enrichment (MEMO) therapy (Gunn-Moore, 2003). Since there is no permanent cure for FIC (Westropp and Buffington, 2016), the main objective of the treatment and managemental changes is to reduce the severity and frequency of recurrent LUTS and to improve the quality of life (Naarden and Corbee, 2020).

A male cat aged 3 years (body weight 3 kg) was presented to the University Veterinary Hospital, Mannuthy with the complaint of anorexia, vomiting, dysuria, haematuria (Fig. 1), pollakiuria and periuria. History revealed that the animal was housed indoors, fed solely on dry diet and used an indoor litter box. There was also a history of construction work in the house in the recent period. The general clinical examination revealed parameters to be within the normal range except for the presence of tachycardia (185 bpm). The physical examination revealed distension of urinary bladder with wall thickening and abdominal pain. The radiographic findings did not show any significant findings except for the distension of bladder (Fig. 2). Ultrasonographic findings included distension of bladder with wall thickening and hyperechoic structures inside the bladder (Fig. 3). The haematological findings were within the normal range. Serum biochemistry evaluation revealed elevated blood urea nitrogen (85.14 mg/dL) and creatinine (8.5 mg/dL) levels possibly resulted from urethral obstruction. Venous blood gas analysis revealed a mild decrease in blood pH (7.2), bicarbonate (16 mmol/L), base excess (- 9.6 mmol/L) and calcium (1.09 mmol/L) along with a mild increase in potassium (5.5 mmol/L) possibly associated with urethral obstruction (Lee and Drobatz, 2003). The electrocardiographic findings were found to be normal except for the presence of tachycardia that might have resulted from the excessive sympathetic system activity developed in response to pain and stress which would have masked the effect of hyperkalemia (Segev *et al*., 2011). Urine samples were collected aseptically by transurethral catheterisation under sedation with the combination of butorphanol (@ 0.3 mg/kg intravenously) and diazepam (@ 0.5 mg/kg intravenously) as suggested by Balakrishnan and Drobatz (2013). The urine sample was subjected to both macroscopic and microscopic evaluation. The macroscopic examination revealed reddish turbid urine with a pH of 7.5 and a specific gravity of 1.030. Microscopic examination revealed presence of erythrocytes, leucocytes, struvite crystals and epithelial cells. The urine culture revealed negative results suggestive of sterile urine. Based on the results of diagnostic tests the case was diagnosed as FIC.

The treatment was initiated by bladder lavage with sterile normal saline as it removes the debris, blood clots and crystals from bladder (Osborne *et al*., 1996). The indwelling catheter was kept in place as it was difficult to relieve the obstruction. Indwelling catheters helps to resolve the metabolic changes associated with acute kidney injury, hastens the repair of urethral injuries if present any, reduce chances for re-obstruction and also helps to retain the normal detrusor function as severe bladder distension could result in detrusor atony. Fluid therapy was initiated with Ringer lactate at @ 10 ml/kg/h intravenously to correct dehydration and mild hyperkalemia (Gunn-Moore, 2015). Since the effects of hyperkalemia did not reflect any change in the cardiac function, fluid therapy along with the restoration of normal urine flow was sufficient to counter the hyperkalemia in the present case. Urine output was monitored and the fluid therapy was repeated accordingly. Balanced electrolyte solutions such as Ringer lactate was preferred over normal saline in cats with urethral obstruction, as studies have reported that there was no difference in the rate of decline of potassium, whereas the acid-base imbalances were corrected more rapidly in cats treated with balanced electrolyte solutions such as lactated Ringer's (George and Grauer, 2016). Proton pump inhibitors (pantoprazole

Fig. 1. Haematuria

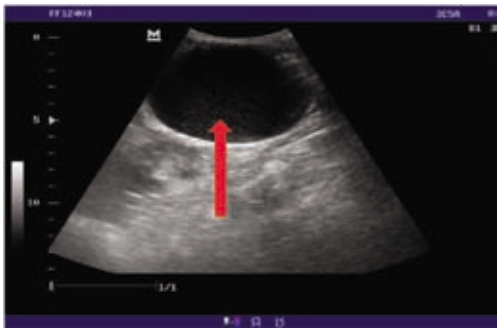

Fig. 3. Distended bladder with thickened wall (2.8 mm) and hyperechoic sediments on ultrasonography

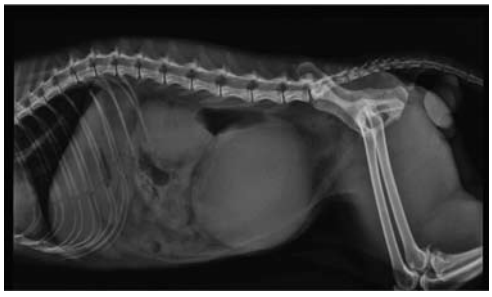

Fig. 2. Distended bladder on lateral survey radiograph

Fig. 4. Improvement noticed after a week of treatment

@ 1 mg/kg q 24 h intravenously), anti-emetics (ondansetron @ 0.5 mg/kg q 12 h intravenously), analgesics (butorphanol @ 0.2 mg/kg q 12 h intravenously) (Westropp and Buffington, 2016) and anxiolytic - glycosaminoglycan supplement (Furinaid plus @ 6 ml/day first 14 days followed by 4 ml/day next 14 days given according to the manufacturers recommendation) were advised. The anxiolytic component in the Furinaid plus was L-Tryptophan, a precursor of serotonin, which would be beneficial in relieving anxiety in cats with FIC (Forrester and Towell, 2015). As per Hostutler *et al.* (2005), the objectives of GAG supplementation was to enhance it's absorption by the damaged urothelium, decrease the permeability of urothelium and to reduce the inflammatory changes. The treatments were repeated according to the response and the animal was evaluated for one week. Significant improvement was observed after a week of treatment (Fig. 4) and the catheter was removed on the seventh day of treatment, followed by repeated urine culture to identify iatrogenic infection if any.

Dietary and managemental changes for stress reduction (MEMO therapy) were instituted as a preventive measure to prevent further episodes of LUTS. Dietary modifications involved changing the diet to a therapeutic urinary diet (Hills c/d Multicare diet) along with managemental changes to increase water intake. Evidence based studies suggest that only therapeutic urinary diets give satisfactory results in FIC (Kruger *et al*., 2015). The managemental changes as a part of MEMO therapy for stress reduction included provision of a core space, avoiding stress from external environment such as inter-cat conflict, guests and babies, provision of enough feeders and waterers, proper management of litter box, improving owner animal interaction and

avoiding sudden changes in routine. Multi-model environmental enrichment therapy was found to be beneficial in cats with FIC as it reduced the concentration of catecholamines, there by reducing the number and severity of recurrent episodes of FIC (Buffington *et al.,* 2006).

Summary

Urethral obstruction is always presented as an emergency. Treatment aims at management of dehydration, metabolic acidosis, electrolyte imbalances such as hyperkalemia and hypocalcaemia, pain relief and relieving urethral obstruction either by catheterisation or surgical procedures. Feline idiopathic cystitis is always a diagnosis of exclusion and the management involves drug therapy, dietary therapy and stress reduction. There is no cure for FIC and the managemental changes are of great importance to prevent the recurrence of LUTS. With dietary management and MEMO therapy, the quality of life can be improved in cats with FIC.

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Conflict of interest

The authors declare that they have no conflict of interest.

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