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Challenges and Advances

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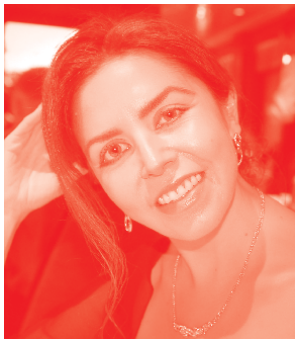

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Edited by Muhammad Abubakar

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Meet the editor

Dr. Muhammad Abubakar, a scientist from the National Veterinary Laboratory, Islamabad, Pakistan, has more than fifteen years of experience in various fields of veterinary sciences. His main area of expertise is transboundary animal diseases (TADs) and antimicrobial resistance (AMR). He has worked in academics as well as in the field of implementing disease control programs. He has worked with local and international projects to establish diagnostic laboratories for TADs. He has also conducted various training for field as well as laboratory staff. Dr. Abubakar has published numerous research papers, review articles, and book chapters on different subjects in the veterinary sciences, especially TADs like avian influenza, foot-and-mouth disease, and peste des petits ruminants. He is the co-editor of *The Role of Biotechnology in Improvement of Livestock*. He is currently an editor in chief for the *Research Journal for Veterinary Practitioners* and *Veterinary Sciences: Research and Reviews*.

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Preface

To recognize and control diseases and disorders in bovines, as well as to improve animal health and production, it is essential to understand recent advances in bovine science. *Bovine Science - Challenges and Advances* presents up-to-date knowledge in the field, covering both introductory topics and more advanced concepts related to bovine health.

The first section is an introduction to bovine sciences for the world economy and food security. It also covers future concepts and perspectives.

The second section on "Advances" discusses new techniques in bovine science and development, such as the use of biogas from bovine confinement.

The third section on "Health Perspective" discusses health and risk factors and diagnosis of disease in bovines. It gives special focus to aflatoxins, which are highly toxic to livestock, poultry, and people.

The fourth and fifth section on "Production" and "Reproduction" examines the economic impact and technologies of bovine production and reproduction.

I would like to express my gratitude to all the contributing authors. My special thanks to Author Service Manager Ms. Jasna Bozic and other staff at IntechOpen publishing for their kind support and great efforts in bringing the book to completion. In addition, I am also thankful to my colleagues, friends, and family for all their support.

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Section 1

Introduction

Chapter 1

Introductory Chapter: Understanding Bovine Science - An Emerging and Re-emerging Menace in the Growing Epoch

Muhammad Abubakar, Zainab Syed and Shumaila Manzoor

1. Introduction

Agricultural sector plays an inevitable role for the economy of most Asian and African countries and is the greatest source of domestic income. In addition, it manipulates 70–90% of the gross working population. Sustainably feeding the world is the crucial challenge in the forthcoming years. Surprisingly, the agricultural sector meets the food needs of 50% families besides income [1]. Livestock animals are important source of food to billions of the world's population. It is also agricultural backbone in developing countries because it adds about half of the value to agricultural output [2]. Livestock contribution to agriculture is 56% among which 11% is imparted to GDP. This escalation is due to increasing demand for livestock product which in turn is driven by growing population size, income, lifestyle change and industrialization in economically developing nations. The need for livestock products is estimated to get doubled by 2030 in unindustrialized countries. However, in technologically advanced countries, the need for such products is comparatively lower and is expected to grow slowly over a specified period of time [3]. Despite the demand variability, livestock dichotomy exists between industrializing and industrialized countries in terms of meat and milk production which are the major commodities obtained from dairy animals globally. Meat is an important source of proteins (myosin, myoglobin and collagen), vitamins (thiamine, niacin riboflavin), iron and zinc etc. Annually, 340 million tonnes meat is produced and the quantity is three times more from the past 50 years. The United States of America (USA) is the world's largest cattle and buffalo meat producing country accounting for 12 million tonnes of meat [2]. Milk, on the other hand is produced by 150 million householders in rural, urban and peri-urban areas and contributes to food security, income and nutrition. The world annual milk production has risen from 530 million tonnes in 1988 to 843 million tonnes in 2018. India is the world's largest milk producing country followed by USA, China, Pakistan and Brazil [4].

Everyone is aware with the importance of agricultural subsector (dairy and livestock industry) in the country's economy; still this sector is prone to variety of emerging and re-emerging threats. The threats associated with ungoverned livestock farming in the form of nutritional deficiencies, metabolic disorders, changing environmental conditions, diseases (either infectious or non-infectious) and antimicrobial resistance has led to the importance of understanding bovine

science. Therefore, in this chapter we will learn about the impact of different kinds of re-emerging and emerging threats on bovine community.

2. Potential constraints to bovine science

Animal health, nutrition, diseases and limited access to available vaccination are the major threats to dairy productivity. Animal diseases directly pose negative effects on economy in the form of reduced milk and meat production, trade restrictions, mortality and animal health issues or indirectly in the form of public health [5]. Livestock bovine is prone to major infectious diseases particularly Foot and mouth disease (FMD), Rinderpest and Brucellosis which has caused previous outbreaks worldwide [6]. Fortunately, by 2010, rinderpest eradication at global level is praise worthy [7]. However, the other diseases are currently endemic in many South-Asian countries including Pakistan. FMD is placed in the list of notifiable diseases by OIE. FMD has also caused epidemics globally and is associated with high morbidity and is a major constraint to economic development in terms of production loss due to changing serotypes [8]. In Pakistan, 200 million dollar loss annually has been reported due to FMD. In India, the loss is even bigger i.e. 430 million dollar. These losses are attributed to poor disease diagnosis and surveillance, lack of reporting and limited data availability on the dynamics and distribution of livestock diseases in South-Asian countries [9].

Several factors are considered as major threats to animal health and livestock sectors. The introduction of new animal in the resident population which has not been vaccinated previously before transportation, is likely to carry infectious agent and cause disease in herd animals. In addition to the factors discussed above, there is a range of threats which could pose dilemmic situation over the livestock herd. If the barriers are removed, then animal's productivity could be improved [10].

Some of the emerging and re-emerging threats are discussed below in detail:

2.1 Nutrition

Various energy proteins, enzymes, vitamins and minerals are needed by animals for their better performance. Nutrients deficiency can have a devastating effect on bovine health [11]. Diet rich in starch concentrates is required by beef cattle for high gains. Besides this, small amount of rough fibrous material should also be included in their diet to ensure efficient rumination.

Gastric or peptic disorders due to heavy rumen acidosis and nurturing of acidogenic products are responsible for 30–42% of cyclic death rates in the feedlots where the overall mortality rates ranges from 0.17% to 0.42% [12]. Moreover, feeding high concentrate diets to livestock bovine has been related with the formation of pyogenic liver abscess and laminitis [10].

Minerals are although required by cattle in a very small amount although, its deficiency might lead to reduced immunity and reproduction of animals. Therefore, for better animal performance and productivity, it is important to improve herd management by increasing the amount and quality of feed produced on cropland, also using feed supplements is suggestive [13].

2.2 Emerging and re-emerging diseases

Emerging diseases can be defined as "any disease which has increased incidence during past couple of decades or is likely to escalate in the forthcoming years". The emerging diseases are fundamentally very important because they are the key players of demographic or social changes alongside encumbrance of illness [14]. Major

epidemics of re-emerging and emerging diseases have been recorded in recent years, most of them are caused by viruses [15].

Re-emerging diseases are those disease which have been managed in the past but there is a chance of their reversal in near times. Different factors play role in the emergence of disease but most likely it is due to host factors, environmental conditions or pathogen adaptation [16]. The emergence and re-emergence of diseases like brucellosis, FMD and antimicrobial resistance can render huge impact on national and international economy, animal and human health [14].

2.2.1 Metabolic diseases

Cattle metabolic disorders are the group of diseases affecting cattle, right after parturition. Different metabolic diseases have been identified in dairy cattle. These include ketosis, udder edema, downer cow syndrome and milk fever. Although these diseases are non-infectious but still they cause heavy economic loss in the form of reduced milk yield and impairment of reproductive system [17]. The impact of these diseases on animal milk production, survival and fertility is of paramount importance to evaluate diagnosis and treatment regimens and prevention strategies because the economic loss caused by these metabolic disorders in dairy farming is poorly addressed [18].

Ketosis refers to the elevated levels of ketone bodies without any clinical signs. Ketosis causes huge economic losses through reduced milk production and other associated pre-parturient disorders [19].

Downer cow syndrome is a cow disease with milk fever in which the cow did not recover from the early recumbence within first 24 hours after intravenous administration of calcium. This disease is also known as 'fat cow syndrome', 'creeper cows' or 'downers'. Downers is characterized by disease complex including milk fever and tendon, nerve or muscle injuries [20].

Milk fever is a metabolic disorder of dairy cows occurring during parturition and lactation period. The disease is also known as eclampsia, parturition paresis, parturient apoplexy and paresis peurperalis. The increasing milk production after calving increases the demand for minerals and glucose at that time when the intake of food have not reached its peak leading to the drainage of calcium and glucose from blood rendering the animal under stress with decrease metabolic activity. Reduced calcium levels are observed before, after or during calving. Accordingly, management of eclampsia is very important economically as it not only results in reduced milk production, but also death of animal [17].

Udder edema is an emerging threat to dairy cow because it has the potential to effect animal welfare and farm's profit. Udder edema may be the result of stress, physiological condition, genetics or nutrition. The disease is characterized by the accretion of lymphoid fluid in the interstitial spaces of mammary gland and their adjacent tissues [21]. Prevalence of udder edema is high in dairy cattle affecting their life negatively. The supporting framework of udder may be broken down due to tissue necrosis [22]. The teats get swollen making their attachment to milking unit difficult resulting in decreased milk production and other secondary infections such as mastitis and udder cleft dermatitis [22]. Severe cases may also lead to farm culling. However, edema can be managed by feeding formulated feed rich in anionic salt, vitamin C, vitamin E, flavonoids and carotenoids (for the management of oxidative stress) [23].

2.2.2 Bovine diseases

The advent of "old-new" animal diseases in the last few years have challenged veterinarians and animal health workers. The list of animal diseases enlarges as

the livestock and dairy industry grows up with adding new research tools and techniques and rapid point of care detection. Consequently, large number of new disease causing agents have been gifted by the veterinary science [24]. They comprise the genetic evolution and emergence of foot and mouth disease virus, bovine brucellosis, bovine viral diarrhea virus and bovine papilloma virus [25]. The reason behind the appearance of these animal disease is difficult to interpret however different molecular factors, genetic evolution, environmental condition and host factors are implicated for playing role in the re-emergence of these diseases. On the contrary, it is implicated that most of the animal disease causing agents are not truly the new agents rather they are the old agents that have been recognized with new technological tools i.e. genomic sequencing. Thus the modern era will keep us bringing robust animal diseases and their associated pathogens [26].

2.2.2.1 Foot and mouth disease

Foot and mouth disease virus (FMDV); an evolving pathogen is an apthovirus belongs to the family picornaviridae. It is a positive-sense, single stranded RNA virus responsible for causing foot and mouth disease in livestock rendering huge economic impact. The disease is prevalent in ungulated animals i.e. cattle, buffalo, sheep, swine and goat [27]. Seven different serotypes (A, O, C, Asia-1, SAT-1, SAT-2 and SAT-3) of FMD are reported from different countries worldwide. It is circulating in about 77% of the livestock population of Africa, Asia and Middle East and is an important transboundary animal disease (TAD). Therefore it can largely disrupt the national and international trade of animals and animal's products and is the leading bottleneck [28]. Disease is characterized by high fever, appearance of blisters (vesicles) in oral cavity (gums, tongue) hooves and teats. The virus initially replicates in the lymphoid tissue of respiratory tract, from there it enters the bloodstream causing viremia. The virus causes death of young animals however it only causes morbidity in infected adult animals and impact can be seen in the form of reduced meat and milk production [29]. Transmission of virus occurs directly through respiratory droplets or it can also occur via mechanical route i.e. fomites [30]. Diagnosis is carried out either by serology (detecting FMD antigen and antibodies) and molecular assay (Real-time PCR) [29]. The FMD is an economically devastating disease, it was initially reported in early 1960s and is still prevalent and endemic in many developing countries. It is estimated that about 75% of the annual cost of low and middle income countries (LMIC) is attributed to FMD prevention and control. The prevention of FMD is based on timely surveillance and implementation of early vaccination programs [31].

2.2.2.2 Bovine viral diarrhea virus

Bovine viral diarrhea virus (BVDV) is a positive-sense, single stranded RNA virus, lying within the genus pestivirus and family flaviviridae. The two predominant species of BVDV is BVDV1 (which possess 21 genotypes) and BVD2 (which possess 3 genotypes) prevailing worldwide [32]. The BVDV subgenotypes BVDV-1a and BVDV-1b and BVDV-1c are distributed widely around the world affecting bovine cattle population and causing severe economic loss in the form of gastrointestinal and respiratory diseases, reproductive disorders, immunosuppressive distress and decreased milk production [33]. The virus has also the ability to cross the placental barrier and cause fetal death andabortion. It can also lead to persistent infection whereby the infected animals can shed virus for the rest of their life [34].

The BVD virus exists in two forms cytopathogenic and non-cytopathogenic based on its ability to produce cytopathic effect in cell culture. BVDV has a high

mutation rate [35]. The occurrence of large number of subgenotypes of BVDV hinder the prevention and mitigation regimes because a vaccine effective in one locality against certain strain is unable to provide immune protection against another strain in another locality [33]. Therefore investigating the number and frequency of subgenotypes of BVDV can be used to study virus evolution which may play role in future vaccination design [36].

2.2.2.3 Bovine papillomavirus (BPV)

Bovine papillomavirus (BPV) is positive sense, non-enveloped, double stranded DNA virus having 8 kb genome and icosahedral symmetry [37]. Virus replicates in the epithelial cells squamous nuclei affecting skin and mucosal surfaces causing malignant or benign tumor. The virus was initially characterized in cattle from where it has got its name [38]. Uptil now, 26 types of BPV has been identified among which three are unclassified and the rest 23 have been characterized into 5 genera: the deltapapillomavirus contain 4 types (BPV-1, 2, 13 and 14). The genus Xipapillomavirus has further 2 species: Xipapillomavirus 1 (BPV-3, BPV-4, BPV-6, BVP-9, BPV-10, BPV-11, and BPV-15) and Xipapillomavirus 2 (BPV-12). Epsilonpapillomavirus 1 contains (BPV-8 and BPV-5) and the last Dyoxypapillomavirus 1 includes (BPV-7). Among the species described, the members of deltapapillomavirus (BPV-1, BPV-2 and BPV-13) has the ability to infect bovine cattle causing bovine papillomatosis [39]. Bovine papillomatosis is characterized by the formation of papilloma or cutaneous warts that is the representative of proliferative lesions ranging from minute lesion to rough, spiny cauliflower shaped warts, often black and gray in color [40]. The virus has caused considerable economic losses previously and currently is causing damage to dairy industry and cattle hides. BPV infects teats and udder of milking cows, and can also cause udder and gastrointestinal cancer. The milking process of young calves is also affected predisposing these animals to secondary bacterial infections. Transmission occurs due to contaminated milking equipment, or animals rubbing them on uncleaned objects (wire fences). Sexual transmission of virus in venereal warts is also recorded [41]. The virus affect cattle of all ages. In the past, detection of virus was carried out using isothermal loop-mediated amplification process but with the advances in the field of science and technology, the in-point of care detection has been replaced by sensitive, fast and reliable molecular techniques i.e. polymerase chain reaction (PCR) [42].

2.2.2.4 Bovine brucellosis

Brucellosis is the disease of cattle, goats, sheep and pigs caused by bacteria, *Brucella melitensis* and *Brucella abortus*. It can spread after having direct contact with the infected animal or animal product [43]. It is classified as category B pathogen by CDC because of its potential to be used as bioweapon in biowar. Although, the disease is prevalent worldwide but its distribution is changing due to newly emerging and re-emerging focuses. This bacteria has the ability to immediately adapt itself to the new environmental conditions and spread after having direct contact with the infected animal or animal product [44]. *Brucella* in animals can cause huge economic damages in the form of spontaneous abortions which in extreme situations are known as *abortion storms*. Pastoralism of cattle, goat, sheep and buffalo have aggravated the occurrence of brucellosis because of the broad spectrum of its transmission in different species [45]. Rose Bengal test, indirect ELISA assay and molecular diagnostics procedures (PCR) are the proposed tests for sensitive detection of *Brucella* in livestock [46]. Effective management

and surveillance strategies, mass vaccination and animal husbandry practices can prevent the transmission of infection [47].

3. Antimicrobial resistance (AMR)

A microbe is known as human's best friend but at the same time is his worst enemy. Antibiotics are known as magic bullets because of its magic in the previous era [48]. Antimicrobial resistance (AMR) can be defined as "the ability of any microbe to become resistant to available antimicrobial therapy to which it was previously found susceptible" [49]. AMR is a problem prevailing worldwide in both veterinary medicine and human sector where antibiotics are used mainly for treating diseases and animal growth promotion [50]. AMR is complex and multifactorial process and is the result of antimicrobial usage (AMU) in animal, human and agriculture sector. The rapid dissemination and spread of resistant bacteria and their associated genes are responsible for increased mortality and morbidity in animals, reduced production and diminished food security [48]. The overuse and misuse of antimicrobial agents has resulted in the appearance of resistance, threatening the prevention and treatment regimens [51]. Antibiotics are also added as food supplements in animal feed which also give rise to resistant bacteria. The usage of many of the growth supplements have been banned in most of the countries especially colistin, because it is the last resort antibiotic and it is also a resurgent drug therefore it can pose great risk of AMR [52]. The WHO (World Health Organization) identified potential antimicrobial resistant agent to which urgent antibiotics are needed [53]. Among the top priority pathogen; extended spectrum beta-lactamase (ESBLs producing *Escherichia coli*) have been recognized as emerging universal dilemma due to their increasing occurrence in livestock sector [54].

Among food-producing animals, cattle are the important reservoir of bacteria capable of producing ESBLs [55]. ESBLs are beta-lactamases that have the ability to degrade ceftazidime, ceftriaxone, cefotaxime, cefepime and monobactam antibiotics. The important variants of ESBLs are TEM, SHV, OXA and CTX-M. Of these variants, TEM and SHV were initially identified in *E. coli* and *Klebsiella pneumoniae*. *Pseudomonas* specie carries TEM, SHV and CTX-M. It is implicated that CTX-M has

Method	Description
Use of eubiotics instead of antibiotics as growth supplements	Eubiotics include prebiotics, probiotics, oil, herbs and enzymes which can be used in animal feed instead of antibiotics. Eubiotics are known for improving performance and health of animals.
CRISPR-Cas 9 system	Gene editing via CRISPR-Cas9 technology can target antibiotic resistant gene and ultimately kill the microbe.
Antimicrobial peptides	Synthetic antimicrobial peptides have broad spectrum of activity against gram positive and gram negative bacteria because they are amphipathic, and rapidly kill the pathogen by depicting lower propensity and toxicity compared to antibiotic.
Bacteriophages	Virulent phages have the ability to disrupt the cell membrane and eliminate the bacteria carrying resistant genes.
Use of conjugated antibiotics	Toxicity, bioavailability, short activity and efficacy are the major shortcomings of naturally occurring antibiotics. Use of conjugated antibiotics can eliminate the resistance forming pathways and can effectively deliver antibiotics to their target portion.

Table 1.
Procedures and their details for combating antimicrobial resistance [57].

been originated from the *Kluyvera* specie persisting in the environment. Most of the bacteria carry the ESBLs on their plasmid facilitating horizontal gene transfer while CTX-M is encoded on bacterial chromosome [56].

AMR is a challenging problem and tackling it is hard however, advances in the field of biotechnology and nano-technology have resulted in procedures which not only target resistant genes but can also be used for treating microbe associated diseases in animals [57]. Many of these procedures are described in **Table 1** given below:

4. Vaccination

Vaccination is an important component for the constructive management of cattle diseases. In-order to ensure effective vaccination of animals, knowledge about the pathogen, its disease-causing ability, host factors, immune status, stress and optimal vaccination timings should be taken into considerations [58].

For the management of respiratory diseases in cattle, modified live-virus vaccines are generally recommended. The live vaccines were available commercially since 1970s. Vaccination is considered to be effective considering it should be biologically active and stimulate active immunity against the pathogenic agent present in the vaccine. It should reduce the clinical illness, improve animal weight and should be economical with long-lasting immunity in commercial setting. However, the existing data suggests that the vaccine immunity gets compromised because during the transportation of animals, the release of pro-inflammatory factors put the animal under stress. Therefore, it would be beneficial to administer vaccine before animal shipment [58].

Excellent animal based surveillance studies along with vaccination might play a crucial role in the management of *Brucella* infection. In case of *B. meletensis*, the two commonly used vaccines are Rev1 and RB51. These vaccines do not interfere with the result of serodiagnosis and therefore can be used in the susceptible animal population for controlling *Brucella*-induced infection and relative abortions [47].

In veterinary virology, both live and killed vaccines are used for controlling livestock diseases, however their efficacy have been compromised due to new and re-emerging virus infections. Therefore, it is necessary to develop new vaccines containing prevailing serotypes and subtypes. Nano-particles have gained considerable fame in veterinary science due to their small size, large surface area and their ability to act as carrier vehicle for antigen delivery and immunogenicity [13]. In case of FMD infection, inactivated vaccines are used mostly but in order to initiate a protective immune response, a time period of almost 7 days is required. Therefore, in order to avoid such situation, silver nanoparticles were used as an adjuvant for the polyvalent inactivated FMD vaccines. Silver nano-particles have been shown to induce a much stronger humoral and mucosal immunity by enhancing interleukin production. Therefore, for long-lasting immunity, silver nanoparticles can be used as adjuvant in vaccine subunits [59]. Further, understanding the molecular mechanism of pathogen and their interaction with host and environment might also be beneficial for future vaccination studies.

5. Conclusion

Management is key to disease prevention and is utmost necessary for global and national food security and animal wellbeing. Dairy cattle and buffaloes play important role in economic development because of their milk and meat production

ability. However, livestock and dairy animals are vulnerable to variety of metabolic and infectious diseases, the introduction of new animal in the herd increases the likelihood of disease. Besides this, antimicrobial resistant strains are also circulating in the environment. The dissemination of antibiotics resistant genes in animals from the surrounding environment poses threat to animal in the form of abortion and reproductive disorder. Procedures to reduce the risk seem plausible. Many live and inactivated vaccines are administered to animals to protect them from diseases. However, these vaccines may not provide lifelong immunity Therefore, a comprehensive surveillance system and mass vaccination programs are needed in light of current dilemma to cope with the scenario.

Conflict of interest

The authors declare no conflict of interest.

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Section 2

Advances

Chapter 2

Mechanical Properties and Elasticity Model for Bovine Hard Tissue

Mrudula S. Kulkarni

Abstract

This chapter aims at establishing engineering material properties of bovine hard tissue cut out of long bone. The study and design of implants, medical devices, and their bone material necessitate the knowledge of mechanical properties of bone to be evaluated. Braces or steel plates are used as fixation devices in animals who are treated for the fracture to bone or cracked bone. Braces or steel plates are fixed to the bone by rods and screws. For checking the stability of these inserted metallic parts, they have to be compatible with bone. The metal and bone form composite action for the load transfer mechanism. To ensure proper biomechanics and design of these inserts and accessories, we need to know the elastic properties of bone. This chapter establishes the modulus of elasticity, poisons ratio of Bovine femur bone. The experimental study establishes the orthotropic behavior of Bovidae femur bone. This experimental research provides comprehensive mechanical properties of Bovidae femur bone, through series of mechanical tests. By performing compression tests on a bone specimen, stress, strain, elastic modulus, poison's ratio, and yielding point of bone are established. The bovine long bone exhibits orthotropic or transversely isotropic nature of femur bone as expected. The data presented here is for samples derived from goat and water buffalo. The solid mechanics approach using stiffness matrix is adopted to establish elastic constants. The data of elastic constants, compliance, and stiffness coefficients obtained can be used for finite element analysis to simulate stability of composite, femur bone, and metallic fixation. The values of compression strength, Young's modulus, Poisson's ratio, and shear modulus are higher for water buffalo male than that of female showing gender difference. This may be attributed to lower bone density in females due to hormone secretion.

Keywords: elastic constants, compressive strength, femur bone, transversely isotropic, orthotropic, poisons ratio

1. Introduction

The ruminant mammals which include sheep, goats, antelopes, bison, African buffalo, water buffalo, wildebeest, impala, and domestic cattle are the members of the biological family Bovidae. Water buffalo species are particularly used for dairy products such as milk, butter, and cheese on large scale. After age, they are also useful for meat. Hence this is an economically important species. Similarly, goats are widely used for meat. In rural India, goats are useful for milk also. In the case of water buffalo, Femoral fractures are observed after falling during mounting or on

slippery flooring. Due to high body weight and an inability to reduce the fracture, Femoral fractures in mature water buffalo have a grave prognosis.

2. Transversely isotropic or orthotropic material properties

In bone, like wood and many other biological structures, there is a "grain" or preferred direction associated with the structure. The mechanical behavior of bone and other directional composites is dependent upon the direction of the applied load. Bone material is assumed as anisotropic, and as many as twenty-one independent elastic constants are required to completely characterize their mechanical behavior. Most materials have planes of symmetry that reduce the number of material constants [1, 2]. For example, materials having properties that differ in each of two mutually perpendicular directions are termed orthotropic. Nine elastic constants are required to fully characterize their mechanical behavior. To determine the nine independent elastic coefficients of an orthotropic material the following mechanical tests are required. Compressive tests in each of three mutually perpendicular material directions; three lateral deformation tests to obtain Poisson ratios. Standards from the ASTM C469, D1621 [3, 4] have been adapted for mechanical testing procedures on biological tissue [5]. Bone is assumed to be highly anisotropic. This anisotropy in different issues may vary. In the long bone appetite needles, collagen fibers, lamellae, blood vassal, etc. show a clear tendency to be oriented along the length of the bone. In general most of the loads in bone are likely to be acting along its length.

3. Composition of bone

Bone matrix is material having both fluid and solid phases. Two main solid phases; the organic and the inorganic (mineral) substance, give bones their hard calcified structure.

3.1 Organic material

Organic matrix consist of type I collagen fibrils and non-collagenous components.

1. Collagen fibrils: The most important factor determining bone tissue quality in terms of its elasticity is collagen arrangement and structure in the bone. Collagen fibrils form 90% of the whole matrix of the bone. Extracellular collagenous matrix is impregnated with inorganic materials, mainly hydroxyapatite Ca10(PO4)6(OH)

2. Non-collagenous components: Non-collagenous components surrounding the mineralized collagen fibers. It is a ground substance, forms the remaining 10% of the organic matrix. It is consisting of protein, Phospholipids, polysaccharides, or glycosaminoglycans (GAGs), chiefly in the form of complex macromolecules called proteoglycans to cement together the layers of mineralized collagen fibers. More information is given by various workers.

3.2 Inorganic material (minerals)

The term mineralized and calcified arises from the fact that the major component of bone is calcium phosphate in the form of crystalline carbonate apatite.

The mineral substance of bone is calcium phosphate hydroxyapatite. On the other hand, they may vary according to the type of bone and may change during the calcification process. In reality, the organic–inorganic relationships in bone are still completely known. Unlike collagen, apatite crystals (Ap) are very stiff and strong. However, bone strength is higher than that of either collagen or apatite, it is because of similar to concrete, the softer component prevents the brittle cracking of stiff one from, while the soft one prevents the stiff component from yielding. The organic material provides bone its flexibility, while the inorganic material provides bone its resilience.

The bone composition depends on many factors, such as the species, type of bone, sample location from which it is taken, and the sex, age, and bone tissue, for example, woven, cortical, cancellous. However, the overall composition roughly estimated by volume is 1\3 rd Ap, 1\3 rd collagen, other organic contents, and 1\3 rd H2O. A roughly amount of Calcium and phosphate is about 65–70% dry weight of a bone. Collagen fibers compile approximately 95% of the extracellular matrix and it is about 25–30% of the bone's dry weight. The amount of water is up to 25% of the total wt. of bone, while 85% of the water to be found in the organic matrix surrounding the ground substance and collagen fibers. The 15% is located in cavities and canals that residence the bone cells.

4. Structure of bone

As described by K. Endo et al. [6], bone is recognized as cancellous, also known as spongy or trabecular and cortical also known as compact. In any long bone, Cortical bone is about four times the accumulation of cancellous bone. The basic material of cancellous and compact bone is identical; thus, the difference between the two is the amount of porosity and the organization. The porosity of cancellous bone ranges from 30 to 90%, while the porosity of cortical bone ranges from 5 to 30%. Bone porosity is not permanent and can alter in response to disease, transformed loading, and the aging process. The periosteum is the fibrous outer covering present in all bones except the joint regions, which are enclosed with articular cartilage. There are various terms used to explain the complex design of bone at a higher resolution. Both cancellous and cortical bone may contain two types of vital architecture, lamellar and woven. Bone can also be termed as primary or secondary bone. The term either haversian or laminar is used for regions within cortical bone. The relative proportion between the compacta and the diverse medulla with the skeletal segments and their role, but the higher strength-to-weight ratio maintains its validity. The calcified volume of thick compacta of cortical bone at the diaphysis of long bones is about 90% and an eccentric cylinder medulla, which containing hemopoietic, red bone marrow in youth, and fatdepleted, yellow, non-hemopoietic marrow in adults. At the flared metaphyseal region the thickness of the compacta is negligible. The short and flat bones, as well as the metaphysis and epiphysis of the long bones, are lined with thin compacta. The medulla consists of interlacing laminar termed, osseous trabeculae.

5. Literature review

Experimental verification of size effects in loaded bovine cortical bone has been carried out by Kieser et al. [7] They represented 2 and 3-dimensional finite element–based numerical models of loaded bovine cortical bone which incorporate the dominant microstructural feature: the vascular channel or Haversian canal system.

The numerical results for the virtual material samples when loaded in bending showed that they revealed size effects not forecast by either classical (Cauchy) or more generalized elasticity theories. The comparison between the values of flexural modulus and characteristic length in bending, for the specimens with axial and transversely orientated voids derived from experimentally measured size effects and those computed with a void fraction of 0.145, SX of 0.5 mm, SY of 0.433 mm, and matrix modulus of 20 GPa was given. They noted the value of axial Young's modulus as17.9GPa and transverse Young's modulus 8.6 GPa. The finite element method showed the value of axial Young's modulus 16.4 GPa and transverse Young's modulus 8.4 GPa.

Wei Sheng et al. [8] and T Attia [9] assessed femur biomechanics of different material assignments. Based on the validity of the assignment using the Finite element method they suggested how to choose the most simple and economic material assignment method. Kaori Endo et al. [6] In this paper they studied the influence of volume of cancellous bone and baseline structure on the variation in cancellous bone strength when subjected to cyclic loading. Two 2-year-old bovines were used to prepare fifteen cubic cancellous bone specimens. They were divided into three groups: femoral head, neck, and proximal metaphysis. Micro-computed tomography was used to determine structural indices of each 5-mm cubic specimen. First samples were subjected to uniaxial compressive loading at 0.05 mm/min with initial 20 N loading, 0.3 mm displacement for five cycles, and then unloading to 0.2 mm with 0.1 mm displacement for five successive cycles. During five loading cycles, elastic modulus and yield stress of cancellous bone decreased exponentially. They correlated the decrease ratio of yield stress clearly with bone volume fraction (BV/TV, $r = 0.96$, $p < 0.01$) and structural model index (SMI, $r = 0.81$, $p < 0.01$). The linking of bone strength after yield stress with structural deterioration of cancellous bone was indicated from data. Finally, they proposed that estimated baseline cancellous bone structure from non-fractured bone contributes to the cancellous bone strength during the collapse. During five loading cycles, elastic modulus and yield stress of cancellous bone decreased exponentially. Yield stress in the bovine femur was Metaphysis, neck and head are 16.8 MPa, 16Mpa, and 30Mpa respectively. Elastic properties were ranging from 428 to 625 MPa.

David C. Kieser et al. [7], Havaldar [10] and Kottha [11] considered cortical and medullary diaphyseal diameters, cortical cross-sectional area, bone length, cortical thickness, and bone density for morphological comparison. The four-point flexure tests for bending stiffness, Young's modulus of bending, and ultimate strength in bending tests was conducted as Biomechanical tests. Mid-diaphyseal cortical compressive elastic modulus and strength for torsional stiffness (Nm/degree) were also studied. Three samples of every bone type

a. rear deer femur;

b. rear pig femur, and

c. rear sheep femur were used for tests.

Young's modulus and ultimate strength in bending for whole bone samples were determined by a four-point bend test of the whole femora. The load was applied through the top rollers, with the lower supporting rollers being self-aligning. The previously reported ultimate strength for deer femora was 174 MPa but they observed a lower value of 98 MPa. For sheep femur, it was 44 MPa.

Mohamed S. Gaith and Imad Al-Hayek [12] compared elastic stiffness and the degree of anisotropy for the femur human and bovine bones is presented.

Orthotropic symmetry is used to model Bovine and human femurs. The mechanical elastic stiffness can be described by nine independent elastic stiffness coefficients which are a function of elastic material parameters, namely, Young's modulus, shear modulus, and Poisson's ratio. The largest value (72 GPa) was noted for bovine plexiform while the human tibia bone has the smallest. The bulk modulus and the overall elastic stiffness have the same behavior for all bones except phalanx. Elastic moduli are an important parameter to expose internal anisotropy and its effect on bonding strength. In conclusion, they stated that the largest overall elastic stiffness observed for bovine femur plexiform and has the most isotropic (least anisotropic) symmetry also seen in bovine [13, 14].

6. Mechanical testing of hard tissue

6.1 Selection of bone sample

The femur bone is selected for the study of its elastic properties. The head of the femur.

articulates with the acetabulum in the pelvic bone forming the hip joint, while the distal part of the femur articulates with the tibia shown in **Figure 1**. By measures, the femur is the strongest bone in the body.

Selection of bovine species:

1. Goat Male (Capra aegagrushircus)

2. Water Buffalo Female (Bubalusbubalis)

3. Water buffalo Male (Bubalusbubalis)

Basic specimen preparation as per standards of materials testing code given by, American Society for Testing and Materials (ASTM). Designations for compressive testing (ASTM C469, D1621) [3, 4].

6.2 Sample collection and preservation

Freshly cut Femur bone samples from Goat Male were collected. All muscles and soft tissue of bone should be removed. Samples to be washed with water and kept in hydrated condition. The weight of the Goat male femur bone was 125gm and length was 18 cm, While the weight of one buffalo male or female femur bone was 1100 gm and the length was 35–40 cm. The diameter of the long bone middle section of female buffalo femur bone was seen to be greater than male buffalo femur bone. But the length of male buffalo femur was found greater than female buffalo femur.

Figure 1.
Test specimen, fresh femur bone of goat male (Capra aegagrushircus).

The sample was preserved in distill water at 24 degrees Celsius. Samples were tested within 5 days.

6.3 Sample cutting and preparation

Yuri 4 inch Cutting Wheel Diamond cutter was used for cutting sample as shown in **Figure 2**. At the time of cutting of sample with a diamond cutter, bone sample reduces its strength and minerals because of high heat formation due to friction at the cut. It finally causes error in results. For good results samples were prepared by using saline water as a lubricant to release heat at the interface of diamond cutter and bone.

6.4 Compression testing of samples

The specimens for compression test are rough-cut cubes out of bone with the orientation of specimens maintained along the axis of the long bone. During the test, bone is kept at room temperature (~24°C) in wet conditions. During testing, care is taken to ensure that the test specimens are kept hydrated. Three cubic samples were obtained from one femur bone. Out of three cubic samples, one sample was kept along the direction of fibers i.e. along the longitudinal axis of bone for compression test as shown in **Figure 3**. The other two samples were used to measure the compression strength of femur bone in the transverse direction shown in **Figure 3**.

6.5 Calculation of properties

To find elastic constants of bone, fundamental elasticity equations were used assuming transverse isotropy of bone. Measurement of longitudinal and lateral

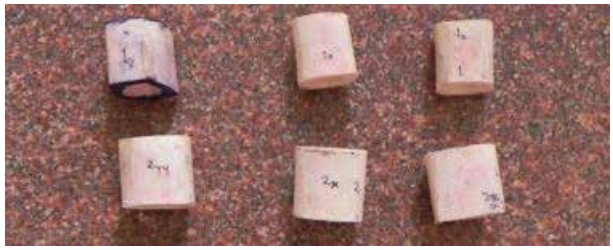

Figure 2.
Test sample, cubes of water Buffalo female (Bubalusbubalis), cut out of test specimen.

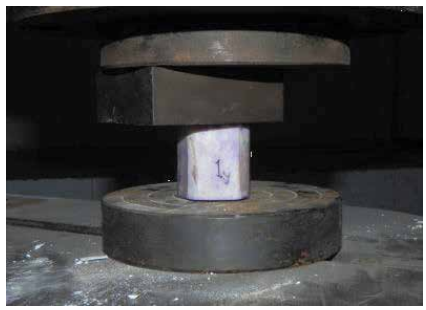

Figure 3.
Testing of the bone sample for compression load, tested under UTM, with controlled rate of loading.

deformations facilitates the calculation of strains in respective directions. Stresses are evaluated from loads applied.

Sample calculation of Goat Male

1. Compression strength (σ) = P/A, where P is the compressive load at failure, A is cross sectional area.

2. Linear strain (εL) = δL/L

3. Lateral strain (εT) = δT/L

4. Young's moduli (E) = σ/εL

5. Passion's ratio (ν) = εT/εL

6. Shear Modulus = E/2(1+ν)

Orthotropic material stiffness matrix constant of Goat Male (*Capra aegagrushircus*)

$$C_{ijkl} = \begin{Bmatrix} 9.08 & 1.81 & 1.85 & 0 & 0 & 0 \\ 1.81 & 9.08 & 1.85 & 0 & 0 & 0 \\ 1.85 & 1.85 & 13.4 & 0 & 0 & 0 \\ 0 & 0 & 0 & 3.57 & 0 & 0 \\ 0 & 0 & 0 & 0 & 3.57 & 0 \\ 0 & 0 & 0 & 0 & 0 & 3.6 \end{Bmatrix} \quad (1)$$

Orthotropic material stiffness matrix constant of Water Buffalo Female (*Bubalusbubalis*) (**Table 1**)

Property	**Goat male (*Capra aegagrushircus*)**	**Water buffalo female (*Bubalusbubalis)***	**Water buffalo male (*Bubalusbubalis)***
σ_1	45 MPa	23.9 MPa	25 MPa
σ_2	46 MPa	24.5 MPa	26 MPa
σ_3	91 MPa	54 MPa	63 MPa
E_1	7923 MPa	5060 MPa	5790 MPa
E_2	8512 MPa	4860 MPa	5660 MPa
E_3	12600 MPa	11080 MPa	11780 MPa
ν_{12}	0.17	0.193	0.205
ν_{23}	0.18	0.211	0.216
ν_{31}	0.19	0.222	0.233
G_{12}	3385 MPa	2126 MPa	2402 MPa
G_{23}	3606 MPa	2008 MPa	2319 MPa
G_{31}	5042 MPa	4540 MPa	4788 MPa

Table 1.
Mechanical properties derived from testing.

$$C_{ijkl} = \begin{Bmatrix} 5.19 & 1.12 & 1.2 & 0 & 0 & 0 \\ 1.12 & 5.19 & 1.2 & 0 & 0 & 0 \\ 1.2 & 1.2 & 8.28 & 0 & 0 & 0 \\ 0 & 0 & 0 & 1.09 & 0 & 0 \\ 0 & 0 & 0 & 0 & 1.09 & 0 \\ 0 & 0 & 0 & 0 & 0 & 2 \end{Bmatrix} \quad (2)$$

Orthotropic material stiffness matrix constant of Water buffalo Male (*Bubalusbubalis*)

$$C_{ijkl} = \begin{Bmatrix} 6.06 & 1.27 & 1.49 & 0 & 0 & 0 \\ 1.27 & 6.06 & 1.49 & 0 & 0 & 0 \\ 1.49 & 1.49 & 12.56 & 0 & 0 & 0 \\ 0 & 0 & 0 & 2.33 & 0 & 0 \\ 0 & 0 & 0 & 0 & 2.33 & 0 \\ 0 & 0 & 0 & 0 & 0 & 2.4 \end{Bmatrix} \quad (3)$$

7. Discussion

This experimental study shows that Young's Modulus and Poisson's Ratio can be determined from load–displacement relation. When tested using UTM, All bone samples show much higher modulus in the axial direction (E_y) than the other two transverse directions, this is due to the transversely isotropic behavior of bone. This is attributed to the parallel orientation of grains along the longitudinal direction. In the other two transverse directions, Modulus was nearly the same. This is consistent with the values obtained by other researchers, Reilly and Burstein [15], Kulkarni and Sathe [16], R. Shahar [17]. Hence orthotropic nature of the material is exhibited by long bone. It is seen that the values of compression strain, Young's modulus, Poisson's ratio, and shear modulus are higher for Water Buffalo Male (Bubalusbubalis) than that of female showing gender difference. This may be attributed to lower bone density in females due to hormone secretion.

The results obtained here for Poisson's ratios of femur bone fall within a narrow range. The values found on our experiments for goat male 0.17 to 0.19, for Water Buffalo Female (Bubalusbubalis) 0.19 to 0.22, and for Water Buffalo Male (Bubalusbubalis) 0.22 to 0.23. These values are in good agreement with 0.20 to 0.22 reported by Kulkarni and Sathe [16], however, the range is different than 0.12 to0.63 reported by Pithioux et al. [18].

The values of compression strength of femur bones tested in this study show variation in three groups of samples. The compressive strength of the Goat male (Capra aegagrushircus) femur bone ranges from 92 MPa to 100 MPa in the longitudinal direction. The compression strength of the Goat Male (Capra aegagrushircus) femur bone reported in this work is 97 MPa. A previous study reported a similar value of compressive strength of femur bone in Ovis (sheep) 90 Mpa, Erickson et al. [19]. The compressive strength of Goat male (Capra aegagrushircus) femur bone ranges from 40 MPa - 52 MPa in the transverse direction.

Considering the above results following conclusions may be drawn:

1. Diameter of the diaphysis of Water Buffalo Female (Bubalusbubalis) femur bone was seen to be greater than Water Buffalo Male (Bubalusbubalis) femur

bone but the stiffness constant shows the higher valves in Water Buffalo Male (Bubalusbubalis) than Water Buffalo Female (Bubalusbubalis) because in Water Buffalo Male (Bubalusbubalis) femur bone grains are closely spaced that is more compact cortical bone and less spongy but in Water Buffalo Female (Bubalusbubalis) femur bone grains are relatively sparsely spaced i.e. It also exhibits more cancellous bone and more spongy nature as compared to male bone sample, this difference is attributed may be due to hormonal effect.

2. In Goat male (Capra aegagrushircus) femur bone compression strength in the longitudinal direction (91 MPa) is higher than that of transverse direction (46 MPa) and the same thing observed in Water buffalo (Bubalusbubalis) species. The higher values in the longitudinal direction than transverse direction is attributed to the orientation of laminas is parallel to the longitudinal direction and hence bone takes more load in the longitudinal direction.

3. For Goat male (Capra aegagrushircus) femur bone Young's Modulus in two transverse directions (E1, E2) is 7.8 GPa and 8.5 GPa respectively which is nearly the same but it has a higher Youngs Modulus value in the longitudinal direction (E3, 12.6 GPa) which indicates that bone is transversely isotropic. While for water buffalo the modulus of elasticity values are observed to be, female in transverse direction 5060Mpa, In longitudinal direction 11080 Mpa. For Male buffalo in transverse direction 5790 Mpa, Longitudinal 11780 Mpa

4. For Goat male femur (Capra aegagrushircus) bone Poisson's ratio varies from 0.17 to 0.19, for Water buffalo (Bubalusbubalis) they are from 0.19 to 0.23.

5. Water buffalo (Bubalusbubalis) has a larger bone cross-sectional area than Goat male and thus stress is comparable to bone cross-section. (Capra aegagrushircus) The magnitude of shear modulus is seen as a higher value in Goat male (3.6 GPa. (Capra aegagrushircus) than Water buffalo (2.0 for Female, 2.4 GPa for Male) (Bubalusbubalis). This is in line with the fact that Goat bone is subjected to more torque due to jumping and twisting and hence exhibits more torsional strength than buffalo, by natural cell development.

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Conflict of interest

The authors declare no conflict of interest.

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Chapter 3

Separation of Bovine Serum Albumin (BSA) Protein by Foam Fractionation Technique

Avishek Mandal

Abstract

Foam fractionation is an effective, low-cost, and environmentally friendly method for water treatment that is widely applied to the removal of hazardous materials, organic materials, and metal ions from the wastewater by using the surfactant as a collector. This type of process known as the adsorption bubble separation technique. It uses bubbles as a separation medium and concentrates the surfactant from its aqueous solution by the difference of adsorption properties of the surfactant on gas–liquid interfaces. During the process of foam fractionation, it spared gas through the bottom of the column to create dispersed rising bubbles. They adsorbed the surfactant onto gas–liquid interfaces of the rising bubbles. We discuss here separate Bovine Serum Albumin from aqueous solution with haemoglobin by foam fraction method. To investigate the effect of the following variables on the enrichment ratio of total protein, the separation process like concentration of feed, the effect of pH, and the Effect of gas flow rate.

Keywords: Concentration of collector, BOVINE SERUM ALBUMIN (BSA), Foam height, Foam density, Foam drainage

1. Introduction

Foam separation, which uses adsorptive bubbles to separate particles, has developed as a viable alternative to conventional separation approaches with ion exchange, chromatography, and precipitation. Foam fractionation is a fast, easy, and efficient method for separating chemical compounds and recovering waste products from aqueous solutions, and it can be used as a pre-concentration method in their analytical determination [1]. The authority takes the waste materials required for reuse in the food industry because of the recycling strategy of various aqueous products. The recovery of whey protein from aqueous products in the food industry is very successful when whey waste is treated as Bovine serum albumin using the foam fractionation technique in batch mode [2]. Its role of pH-induced structural change in interface-induced protein aggregation was investigated using bovine serum albumin (BSA) as a model protein to reduce protein aggregation in foam fractionation. Foam fractionation is a well-known protein purification method that may be useful in the early stages of recombinant and other proteins' downstream production. The process has many benefits, including ease of use, technical simplicity, and hence low cost as opposed to other purification processes. While

much research has been done on particular protein solutions, there's been less done on protein mixtures in order to purify one protein ingredient from a mixture of proteins [1]. In mineral flotation, foam fractionation or adsorptive bubble separation methods have been commonly used. The methods were focused on the surface tension differences between the products to be isolated [3]. This method is also used in the discharge of wastewater. Since organic compounds have a low surface tension and can be enriched at the air-water interface, it is possible to recycle or remove dilute organic compounds found in industrial waste water. Despite the fact that this method has been around since the beginning of the century, more research is needed to further understand the conditioning and viability of proteins and single protein fractions using foam fractionation by concentrating on the most critical process parameters [2]. Solvent sublation is a non-foaming adsorptive bubble procedure that can remove trace amounts of non-volatile and unstable organic materials from wastewater [4]. This approach is also useful for waste water recovery and the elimination of hazardous materials. Solvent sublation has the benefit of achieving better removal efficiencies than bubble fractionation or air stripping. Since the disposal of pharmaceutical unit effluent is a required operation, this method may be used to isolate drugs from waste water released by pharmaceutical industries, thereby reducing harmful pollution.

2. Adsorption bubble separation technique

The term "adsorptive bubble separation technique" was developed by Lemlick et al. who called it the "Adsorption bubble technique." The disparity in surface behaviour is the basis for this technique [5]. The disparity in surface behaviour is the basis for this technique. Material of various sizes, including molecular, colloidal, and macro particulates, is selectively adsorbed or added at the surface of growing bubbles in the liquid, and thereby accumulated or isolated. A material that is not surface-active will often be rendered surface-active by combining with or adhering to a surface-active collector. Colligend is the name for the material that has been extracted. Foamate is a small amount of collapsed foam that is used to concentrate or separate the material [6]. This makes the adsorption bubble isolation approach applicable to a broader variety of compounds, such as ions, molecules, precipitate, active carbon, nano particle, proteins, and bacteria [6].

3. Characteristics of an adsorptive bubble column

Porous frit creates bubbles on a continuous basis, providing a broad surface region for substance movement from the bulk solution. The bubble diameter is observed to be a high function of the orifice diameter and a weak function of the gas velocity in the orifice at low gas velocity (0.5 cm/sec) [7]. The diameter of a bubble at high gas velocity is determined by the gas flow rate. It's important to note that the presence of an electrolyte in a solution has an effect on the bubble scale. In the presence of electrolytes in the water, smaller bubbles form due to surface tension and the electrostatic potential of the resulting ions at the gas–liquid interface. The size of the bubble is determined by the electrolyte concentration and form. Foam Separation and Non-Foaming Separation are the two major types of adsorptive bubble separation methods. To take away liquid, foam separation necessitates the development of a foam or froth. The separating of products using a non-foaming

process would not necessitate the use of foam [7]. Bubble fractionation and solvent sublation are two subsets of the Non foaming Adsorptive Bubble Separation Process. The movement of fluid inside a liquid by adsorption or addition on the bubble surface, accompanied by deposition of material at the top of the liquid as the bubbles leave, is known as bubble fractionation. Solvent sublation refers to the transition of substance from one miscible liquid to another, or from one miscible liquid to an immiscible liquid mounted on top of the main liquid. Foam fractionation and froth flotation are two different types of foam separation. The foaming off of dissolved content from a solution through adsorption at the bubble surfaces is known as foam fractionation. The removal of particulate material by frothing is known as froth flotation. Based on the material, which can be molecular, colloidal, or microparticulate, froth flotation can be separated into many branches. Mineral extraction from ores using froth flotation in a special situation. Macroscopic particles are separated using macro flotation. The separation of microscopic particles, especially colloid or microorganisms, by foaming is known as micro flotation. Precipitate flotation is the practise of removing a precipitate using a surfactant that is not the precipitating agent. Ion flotation is the isolation of surface inactive ions by foaming with a catcher, which produces an unsolvable liquid, especially whenever the substance is extracted as scum. Molecular flotation, on the other hand, is the isolation of surface inactive molecules by foaming with collectors and resulting in an insoluble substance. Finally, adsorbing colloid flotation is the separation of a solute through adsorption on colloid particles followed by flotation. The following methods can be used to operate the Adsorptive Bubble Separation Process [8]. (a) Simple continuous flow, (b). Simple Batch, (c) Combined enriching and stripping, (d) Continuous flow stripping, (e) Continuous flow enriching by reflux, (f) Staged operation [8].

4. Classification of adsorptive bubble separation method and classification

4.1 Collectors and mechanism of action

When the particles of interest (colligend) do not have surface active properties of their own, they are made effective surface active or collected by adding to the collectors in an adsorptive bubble separation process. Ionic surfactant travels to the surface of gas bubbles increasing through the liquid during ion flotation, and the surface becomes charged [8]. The oppositely charged substance of interest (Colligend) adsorbs to the bubble interface as a counter ion, forming an electrical double layer. Because of the foam's high surface area to liquid volume ratio, the liquid that emerges from its breakdown is much more concentrated in the ion than the original solution. The colligend should be specific for the interface of a charged surfactant. Surfactant molecules have two parts: one is hydrophobic, while the other is hydrophilic. The hydrophilic portion comes outside, while the hydrophobic part stays within. The hydrophilic component was applied to the substance of interest and taken away at the foam bubble interface (**Figure 1**).

Enantiomeric mixtures are separated using chiral collectors. These chiral molecules are known to interact with analytes in a variety of ways, including ligand exchanged interactions, hydrophobic inclusion complexation, and hydrogen-bonding interactions. Colligend must be used as a foaming agent so chiral collectors are not foaming agents. Enantioselectivity can be caused by a disparity in the chiral collector's and two enantiomers' reaction mechanism.

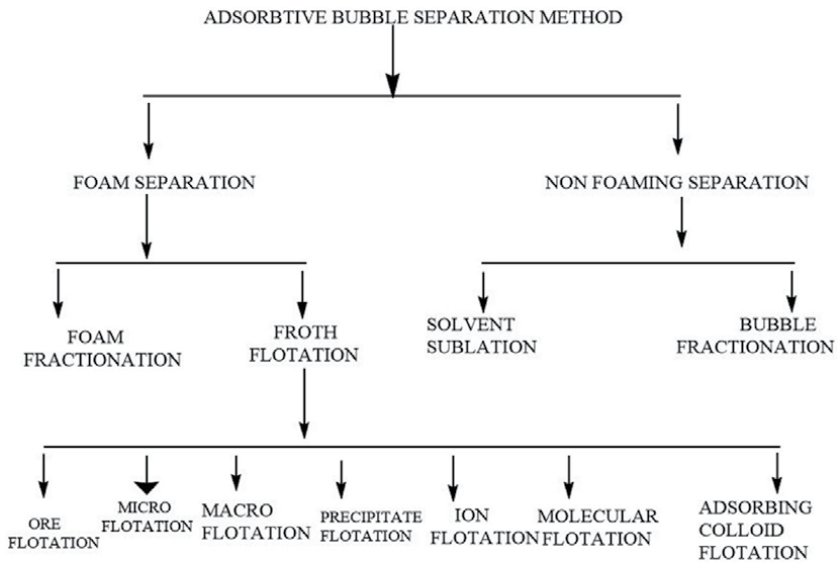

Figure 1.
Classification of adsorptive bubble separation method classification.

5. pH's impact

Ion flotation is especially vulnerable to some of these parameters because variations in pH have a significant impact on the nature and charge of both collector and colligend. Several researchers mention some part of this dependence; the following are the consequences that can be observed when the pH is changed. Hydrolysis or the forming of other complexes can cause a change in the charge on the colligends. Ionisation of the collector can change; acids and amines, for example, can lose their charge at low and high pH values, respectively. They either stop being collectors or change their collecting mode. If ion flotation is preferred, the colligend can be precipitated as a hydroxide and then extracted by precipitate flotation instead of ion flotation. If ion flotation is desired, a difference in the pH can result in a change in the design of the method. The enhanced ionic strength that occurs when the pH is adjusted to extreme values will suppress flotation. The consistency of the foam that supports the sublate could deteriorate, resulting in re-dispersion.

6. Temperature

When the foam durability of surface-active materials varies with temperature, temperature has been proposed as an operating variable. If the binding of the collector to the mineral surface is due to physical adsorption, surfactant adsorption and hence flotation could be assumed to decrease with an increase in temperature in the case of froth flotation of minerals. If somehow the adsorption is caused by chemical forces between the surfactant and the mineral particles, the result could be the reverse. Temperature, on the other hand, was observed to have no impact in the ion flotation and foam fractionation processes in many cases.

7. Gas flow rate

The removal of dissolved compounds is heavily influenced by the gas flow rate, although steady state removals are unaffected. The distribution or division of

dissolved substances between the gaseous and aqueous phases is needed for their removal. The volume and size of gas bubbles, which increase interfacial space, cause an increase in removal at any given moment, depending on the gas flow rate. Low enrichment, on the other hand, increases as the loss of solution increases with a high gas flow volume. Of course, there must be enough gas flow to sustain the foam height, which is necessary for effective separation. The maximum flow rate, on the other hand, is calculated by the surfactant concentration and the foam's nature.

8. Other auxiliary reagents are present

For better separation, many reagents are successfully used in foam separation techniques. In some situations, the results are due to particulate flocculation or collector activation for increased adsorption. Alum, ferric salts, and organic polyelectrolytes are the most widely used flocculation agents in foam separation. Using these flocculating reagents, for example, improved the removal of phosphate and suspended solids from waste water. Activators that facilitate preferential adsorption of the collector on a specific material are often used in the foam separation process.

9. Surface area of bubbles

Spargo and Pinfold made an assumption, which Lemlich et al. addressed. In the flotation cell, they used a pore diameter of 10 μ for the frit. The smallest diameter of this method was 10 μ diameter, and the overall size was about 40 μ. As the flow rate grew, these values increased as well.

10. Foam height

The isolation of albumin is influenced by the foam height, with the effect being more noticeable near the foam liquid interfaces. The foam stream changed dramatically as the foam height was increased from 3 to 17cms. The amount of the solution taken away in the shape of foam was 24 ml/min at a foam height of 30cms, and 10 ml/min at a foam height of 17cms. Furthermore, it was discovered that increasing the foam height resulted in a slight reduction in the process' productivity. At good operating conditions, the height of the transfer unit (HTU) varies slightly with column length for counter current lengths of 10 to 28cms.

11. Foam density

The density of the foam is critical to the operation's progress. When the foam concentration is too high, the bubbles cannot rise to the surface as a result of the pressure, resulting in no separation. High densities of bubbles are seen when there is a large concentration of molecules in solution as well as a large concentration of collections.

12. Foam drainage

While doing a foam separation, the extract must be condensed to a minimum amount as necessary. Foam drainage is usually accomplished by forcing foam upward across a stretch of extended column diameter. Sweitzer P.A. et al. designed

a foam separating mechanism that allows the foams to migrate almost horizontally. He discussed about how this system is better than the vertical system. To begin with, the vertical portion of gas velocity is reduced to 0, and the distance from which the liquid must drain can be rendered uniform and set at any desired value. Second, laboratory experiments with static foams will estimate the drainage that occurs.

13. Equipment design

One of the most important aspects of this method's performance is its equipment configuration. As currently mentioned, there should be enough foam height to provide dry foam, the width and height of the column should be in a specific proportion for liquid delivery, and the frit pour size should be kept to a minimum to ensure enough bubble surface space.

14. Area of potential use in the pharmaceutical field

Purification of products from a combination of ingredients or isolation of drug components. This process isolation of pharmacological activities from plant sources, such as alkaloid mixture salts, separation from soap, and extraction of active components using the foam fractionation process. Enrichment of plant proteins and foam fractionation of fruit juice enzymes, such as **Bromelain** from pineapple, using an adsorptive foam separation process. Finally, pharmaceutical products are removed from waste water.

15. Experimental procedure

The target concentration range of 3 mg/ml was obtained by weighing and dissolving the necessary amount of crystal BSA in double distilled water and then diluting it properly. The optical density (OD) was then measured with a UV-spectrophotometer at a wavelength of 280 nm. A regular curve was created by plotting the resulting value against concentration. The SDS-PAGE system was used to quantitatively estimate individual components in a protein mixture.

16. Determination of critical micelle concentration (CMC) of BSA

Required quantity of powdered BSA was weighed and dissolved in double distilled water and then suitably diluted to obtain a desired concentration range of 100–800 mcg/ml. The surface tension of those samples was measured by using drop count method, and a plot of Concentration VS Surface tension was constructed (**Figure 2**).

17. Measurement of the gas percentage hold up

Different concentrations of feed are prepared for deciding percentage gas holdup while keeping the same Protein surfactant ratio. As gas went through the column, the percentage gas hold up was determined in a batch of oil. The liquid pool's height was assessed. After turning off the gas supply, the height of the liquid

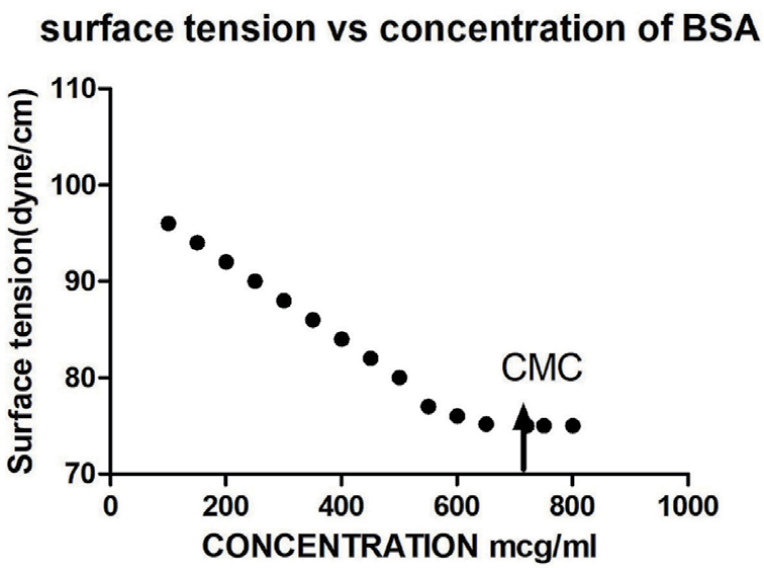

Figure 2.
Surface tension vs. concentration of bovine serum albumin (BSA).

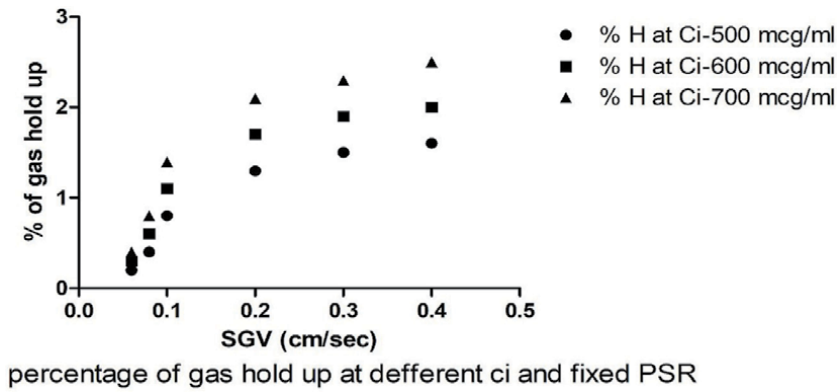

Figure 3.
Percentage of gas hold up at defferent ci and fixed PSR.

pool was measured once more. The percentage of entrapped gas bubbles in the liquid column was measured and tabulated as percent gas hold up. The percentage gas hold up was plotted against the gas flow rate (GFR). The temperature in the lab was held at 25°C (**Figure 3**).

18. Foam fractionation (batch process)

Initially, feed at a certain concentration was made by diluting stock whey with water to the desired concentration. To achieve the correct Protein Surfactant Ratio (PSR), the necessary amount of Sodium Lauryl Sulphate (SLS) was applied to the feed, which was then allowed to mix evenly with the help of an ultrasonic cleaner. The pH of the feed was then calculated and modified as required by adding

concentrated HCl or concentrated NaOH solution. After that, one litter of feed solution was added to the foam fractionation column, and nitrogen gas was moved through the feed at the required gas flow rate (GFR). An analysis of the percentage of gas hold up' The GFR was held between (100 to 200) cc/min. The frit first creates bubbles, which then rise through the liquid column. When bubbles leave a material, they form foam. Foam was absorbed constantly through the top outlet into a receiver as it moved up the column due to gas velocity. A foam breaker was added to the receiver. For the necessary amount of time, the foam was continuously extracted. The foam was then able to collapse using a stirrer until it broke down into foamate (collapsed foam). The total amount of foamate was weighed, diluted appropriately, and absorbance was recorded. The gas was turned off after the procedure was completed, and the residual liquid in the column was extracted. The volume of the residual liquid was calculated, as well as its concentration. The mass flow rate (MFR) was then calculated using a regression equation derived from a plot of protein volume versus time. Enrichment ratio (ER), separation ratio (SR), and recovery percent (percent RP) were all measured as performance parameters for foam fractionation.

19. Foam fractionation (continuous process)

Feed was prepared by suitable dilution of Bovine Serum Albumin (BSA) to get the desired feed concentration. Required quantity of Sodium Lauryl Sulphate (SLS) was added to the feed to get the desired PSR, it was then allowed to mix uniformly with the help of an ultrasonic cleaner. Then the pH of the feed was measured and adjusted as per requirement. The foam fractionation column was then filled with 1 lit. of feed solution and Nitrogen gas was passed through the feed at desired gas flow rate (GFR). Feed was introduced from outside through an inlet into the column with the help of a peristaltic pump to maintain a constant volumetric flow rate (VFR), and the flowing effluent is constantly collected at intervals through a outlet from other side, the flow rate of the outgoing effluent is same as the incoming feed. Bubbles are formed initially which then rises to the top of the column leading to formation of foam. The foam is continuously collected for required period of time. Foam was then allowed to stir using a stirrer until the foam breaks down to form foam. The effluent was collected in a reservoir, the residual was also collected, then the collected material (effluent) was pumped into the second column, where it acts as feed for the second column. When the work with the first column is finished the gas flow into the first column was stopped and the valve is opened so that the gas now flows into the second column and samples were withdrawn at regular intervals assessed. After steady state was achieved, the effluent showed constant concentration. Whole procedure is repeated again as mentioned above. The volume of foam is measured, sample was suitably diluted and absorbance is noted. The total effluent and residual were collected and absorbance was noted, the total input amount, output amount, loss amount, recovery %, enrichment ratio was also calculated.

20. Results and discussion

Binary protein mixtures were used in batch foam fractionation experiments to assess the enrichment ratio and percentage recovery for various feed concentrations at varying pH of the solutions, liquid pool heights, and air circulation rates. The obtained findings are discussed elaborately. The ratio of the concentration of the

foam (CP) to the concentration of the feed liquid (Cf) from which the foam was produced is known as the enrichment ratio or separation factor (E).

$$\text{Enrichment ratio (E)} = \text{Concentration of Foam (Cp)}/\text{Concentration of the feed solution (Cf)} \quad (1)$$

$$\text{Percentage removal (P.R\%)} = \text{Amount of recovered (Cf–Cb) x 100}/\text{Amount of metal ions in feed solution (Cf)}, \quad (2)$$

where Cb is the concentration of metal ion in residual solution.

20.1 Statistical analysis

Each experiment was at least three times repeated. Graph Pad prism 5 was used to do an analysis of variance on the data. The t-test with ($P < 0.05$) was used to determine the difference between mean values.

20.2 The impact of airflow rate

Experiments were carried out with different air flow concentrations at fixed other conditions such as a 30 cm liquid pool height, a feed concentration of 3 mg/mL of Bovine Serum Albumin (BSA) and 2 mg/mL of Haemoglobin, a feed pH of 5.0, a drainage time of 4 minutes, and a foam height of 35 cm. The findings for the impact of air flow rate on enrichment ratio are as follows: The separation factor or enrichment ratio decreased from 2 to 0.568 as the air flow rate rose from 0.3 to 0.8 lpm. Brown et al. verified these findings. The enrichment ratio and percentage elimination increase as the air flow rate is increased from 0.4 to 0.6 lpm at first. However, as the air flow rate increased, the enrichment ratio and percentage elimination decreased. This is because, at low flow speeds, the bubble sizes are greater at first, resulting in more coalescence and drainage. As a result, the enrichment ratio and percentage elimination initially improved. As the air flow rate was increased higher, the foam bubble size declined, and coalescence and drainage reduced. As a result, both the enrichment level and the amount removed decrease.

20.3 Height of the liquid pool impact

Figure 4 shows the effect of liquid pool height on foam concentration and protein enrichment ratio at set other conditions of 0.2 Lpm air flow volume, 3 mg/mL Bovine Serum Albumin (BSA) and 2.0 mg/mL haemoglobin, 5.0 pH of feed, 4 min drainage time, and 35 cm foam height. The enrichment ratio of metal ions improved as the height of the liquid pool increased from 5 to 25 cm, as seen in the **Figure 4**. When the liquid pool's height is greater, the residence time of bubbles in the liquid pool is longer. This results in a higher enrichment of metal ions on the bubble surface, and it may reach a point where enrichment cannot increase much more. This equilibrium is achieved in the current analysis at a pool height of 25 cm.

20.4 Effect of foam height

Figure 5 shows the experimental findings for the effect of foam height on foamate concentration and enrichment ratio. When the foam height is raised from 35 cm, the foam residence time increases, allowing for more liquid draining in the films. As a result, the foam is drier and the enrichment level is higher. The bubble

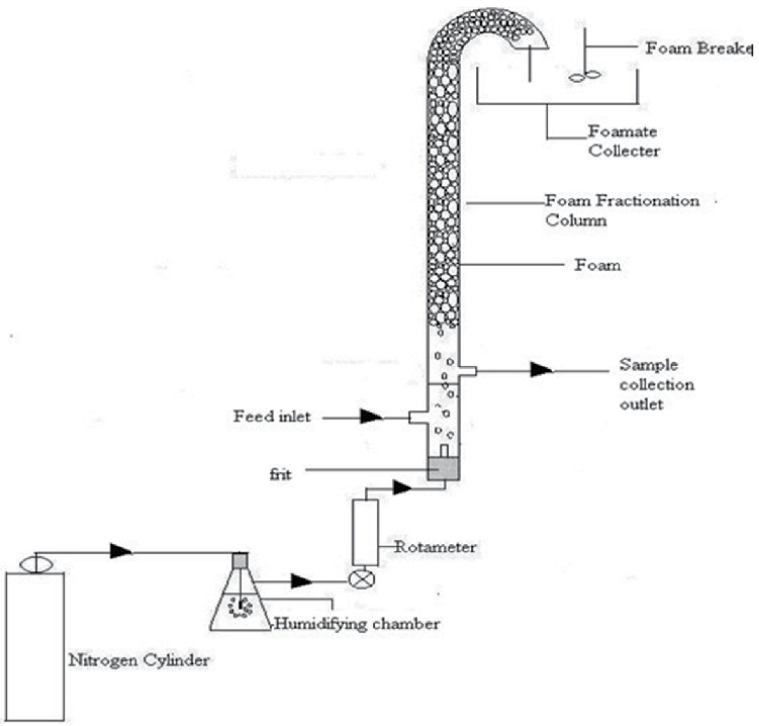

Figure 4.
Schematic diagram of foam fractionation apparatus operating (batch process).

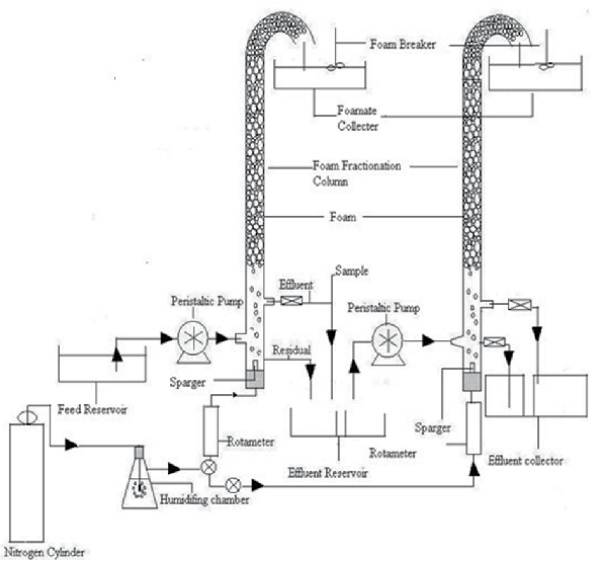

Figure 5.
Schematic diagram a foam fractionation apparatus operating in continuous mode.

scale was found to be larger at the liquid-foam interface, indicating that the drainage is greater. Dry foams appear at the tip of the foam as the height is raised, indicating that optimum draining has already occurred. As a response, no substantial difference in enrichment ratio was observed above a certain foam height (**Figures 6–8**).

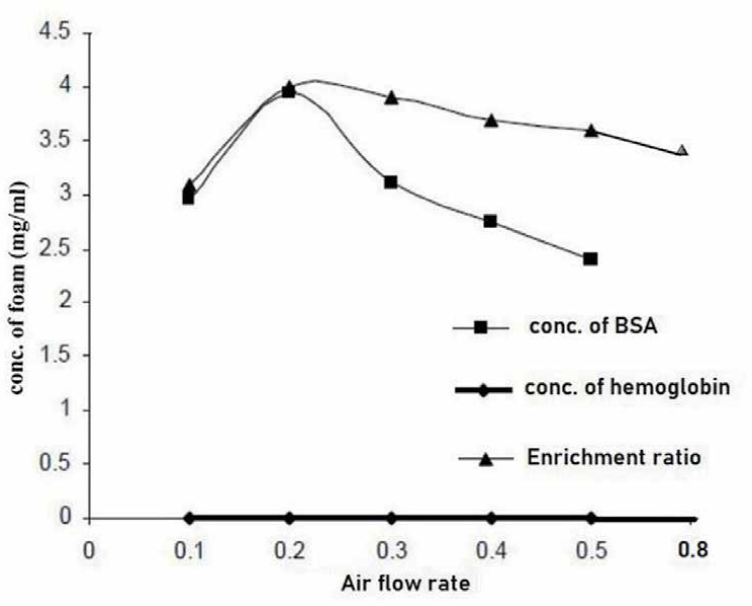

Figure 6.
*Effect of air flow rate on foam concentration and enrichment ratio [liquid pool height = 30 cm, feed concentration = 3 mg/mL of BSA and 2 mg/mL of haemoglobin, pH of the feed 5, drainage time = 4 min, foam height = 35 cm]. Compared to the enrichment ratio of total protein with concentration of BSA in foam concentration, the p-value significantly changed (**$p < 0.05$). As we air flow rate increases so the foam bubble size reaches big resulting in the waste product produce so significantly change observe 0.1 lpm to 0.8 lpm (litre per minute) height increase.*

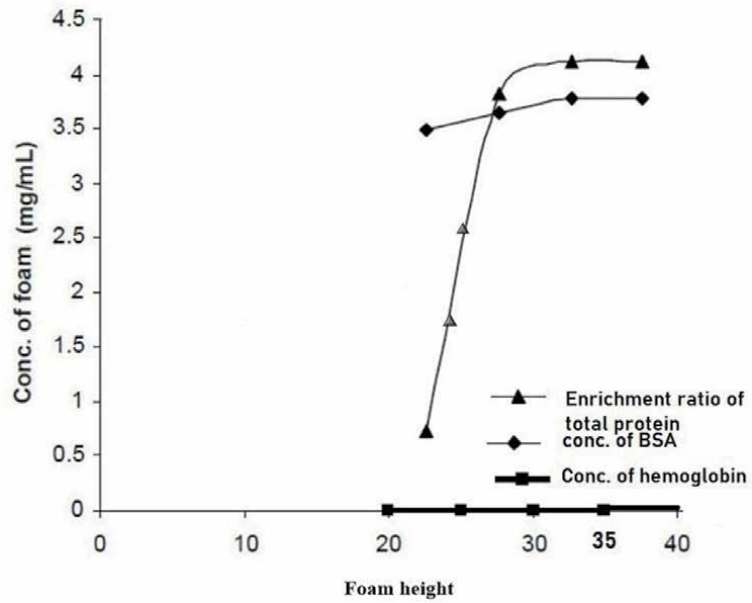

Figure 7.
Effect of liquid pool height on foam concentration and enrichment ratio [air flow rate = 0.2 lpm, feed concentration = 3 mg/mL of BSA and 2 mg/mL of haemoglobin, pH of the feed 5.0, drainage time = 4 min, foam height = 35 cm]. Compared to the enrichment ratio of total protein with concentration of BSA in foam concentration, the p-value significantly changed ($p < 0.05$). As we foam height increase so the foam reaches dry so significantly change observe a 20 cm to 35 cm height increase.

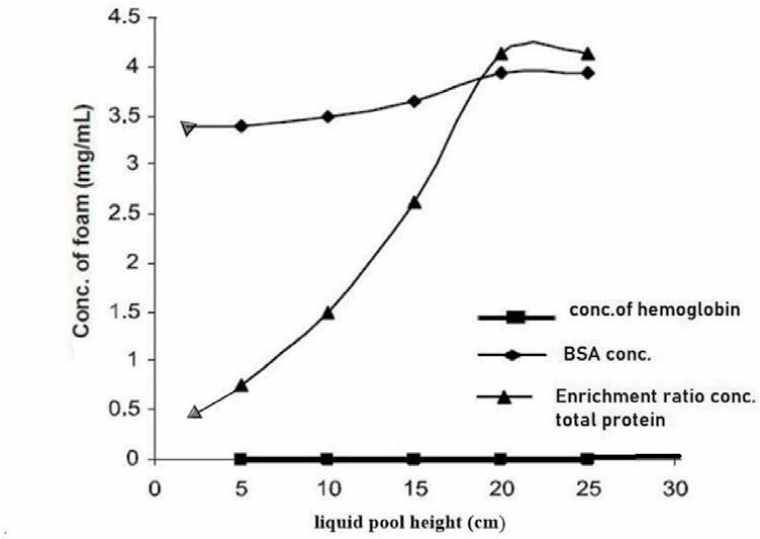

Figure 8.
*Effect of foam height on foam concentration and enrichment ratio of total proteins [air flow rate = 0.2 Lpm, liquid pool height = 30 cm, feed concentration = 3 mg/mL of BSA and 2 mg/mL of haemoglobin, pH of the feed = 5.0, drainage time = 4 min]. Compared to the enrichment ratio of total protein with concentration of BSA in foam concentration, the p-value significantly changed (**$p < 0.05$). As we liquid pool height increase so the foam reaches dry so significantly change observe a 5 cm to 30 cm height increase.*

20.5 Effect of pH of feed

In **Figure 5** the impact of feed pH on formats concentration and protein enrichment ratio is seen. It can be shown that the highest enrichment ratio is obtained at a pH of 5.0. These were induced by the protein's increased hydrophobicity at its isoelectric point. Between proteins adsorbed on the air-liquid interface, an electrostatic repulsive force and the Vander Waals attractive force act. The dissociation of amino acid residues causes the surface charge on the protein molecule. Because this electrostatic repulsion between protein molecules adsorbed on the bubble surface is thought to be lowest at the isoelectric point, proteins should be adsorbed more compactly on the bubble surface at that point (**Figure 9**).

20.6 Effect of feed concentration

Impact of haemoglobin and BSA concentrations in feed on foamate concentration and enrichment ratio seen changes. The concentration of haemoglobin in the feed solution was increased from 0.6 to 1.6 mg/L by maintaining the BSA concentration at 3 mg/L, and it was discovered that as the concentration of haemoglobin in the feed solution was increased, its adsorption decreased, but BSA adsorption increased. That's because the inclusion of haemoglobin in the bulk solution facilitates the adsorption of BSA on the bubble surface. In the presence of haemoglobin, the BSA concentration in the foam is clearly increased. As the feed haemoglobin concentration exceeds 1.5 mg/mL, it ceases adsorbing on the bubble surface entirely. The enrichment ratio and the foam concentration of BSA decrease as the feed concentration of BSA is increased from 2 to 2.9 mg/mL at fixed other conditions of 2 mg/mL of haemoglobin in feed, 0.2 lpm of air flow volume, 30 cm of liquid pool height, 5.0 pH of feed, drainage time of 4 min., and 35 cm of foam height. This may be because the surface tension reduces as the feed concentration

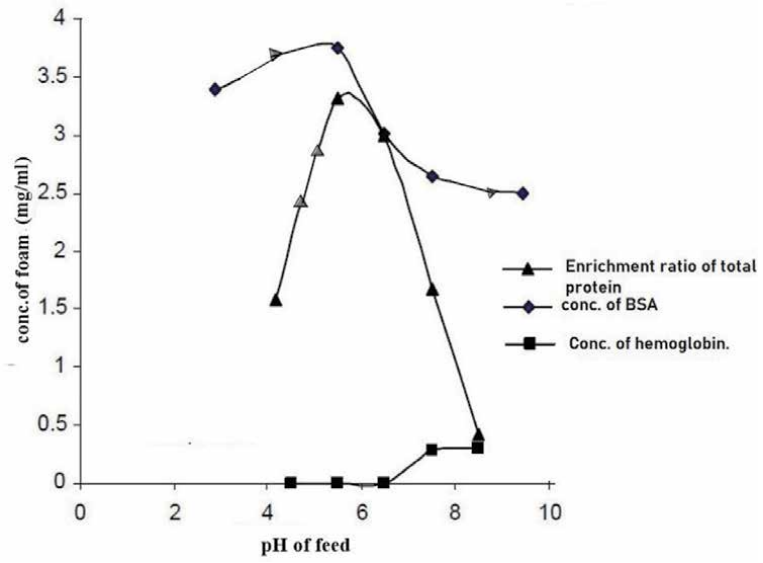

Figure 9.
*Effect of pH of feed on foam concentration and enrichment ratio of total protein [air flow rate = 0.2 Lpm, liquid pool height = 30 cm, feed concentration = 3 mg/mL of BSA and 2 mg/mL of haemoglobin, drainage time = 4 min, foam height = 35 cm]. Compared to the enrichment ratio of total protein with concentration of BSA in foam concentration, the p-value significantly changed (***$p < 0.05$) from pH 4 to pH 8.*

increases. As a result, more stable bubbles form with less coalescence, resulting in decreased drainage. As a result, the wetness of the foam is greater, lowering the enrichment ratio and percentage reduction.

21. Conclusion

The effects of parameters like air flow rate, liquid pool height, feed concentration, pH of the feed, and foam height on the foam concentration and enrichment ratio were studied in experimental studies on batch foam separation of binary proteins such as Bovine serum albumin (BSA) and haemoglobin. The perfect pH for maximal separation was discovered to be 5, perhaps owing to enhanced hydrophobicity of proteins. The amount of BSA adsorbed on the foam increases as the concentration of haemoglobin in the feed increases. This is because the inclusion of haemoglobin in the feed liquid enhances the adsorption of BSA on the bubble surface. At the optimal operating conditions of 0.2 Lpm air flow rate, 30 cm liquid pool height, feed concentration of 3 mg/ml BSA and 2 mg/mL haemoglobin, 5.0 pH of feed, and 35 cm foam height, an enrichment ratio was achieved. As a result, the foam separation technique of pure BSA focused on the foam will successfully separate binary proteins Bovine serum albumin (BSA) and haemoglobin.

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Chapter 4

Biogas Generation from Bovine Confinement: An Energy Policy Option for Brazil

Alexandre Louis de Almeida d'Avignon
and Gustavo Abreu Malaguti

Abstract

Brazil has the largest commercial beef herd in the world and is one of the most important players in the global agricultural market, notably for soybeans. Agriculture has an important demand for energy and emits significant quantities of greenhouse gases (GHG). To minimize the effects generated by livestock activities, from both the energetic and environmental perspectives, there exists the possibility of the use of biogas generated from beef cattle confinement. This productive system allows the reduction of methane emissions from enteric fermentation and from manure through the production of biogas. This has become an option for energy policy by contributing to the offer of energy and the reduction of the demand of agriculture for fossil fuels. With a renewable energy resource, the agricultural sector dependent on non-renewable resources, also reduces its dependence on exhaustible resources, so that a policy aimed at the use of biogas and partial energetic autonomy becomes strategic for the sector. The article analyses biogas production potential from waste throughout the entire beef production chain in more intensive systems (total or partial confinement of beef cattle). These solutions can contribute both to the offer of electric energy to the agricultural sector in the country, increasing its productivity, and to the reduction of greenhouse gases.

Keywords: Bovine confinement, GHG emissions, biogas, energy policy

1. Introduction

With one of the largest cattle herds in the world, with almost 215.2 million heads[1] according to FAO [1] and IBGE data [2], with almost 90% of this being beef cattle (approximately 193.5 million heads of cattle) and almost 22 million head of dairy cattle, in 2015, the production of beef and dairy products, largely based on extensive systems, has a significant environmental impact, including with a large demand for energy resources, most of which is non-renewable and of fossil origin. The **Figure 1** shows countries with the largest cattle herds, 2000-2014.

[1] Emissions generated by other animals will not be analyzed, only those from the raising of cattle. In the case of analyses and the scenarios proposed, the author uses data for the confinement of beef cattle, not considering the herd of dairy cattle which belong to the entirety of the bovine herd.

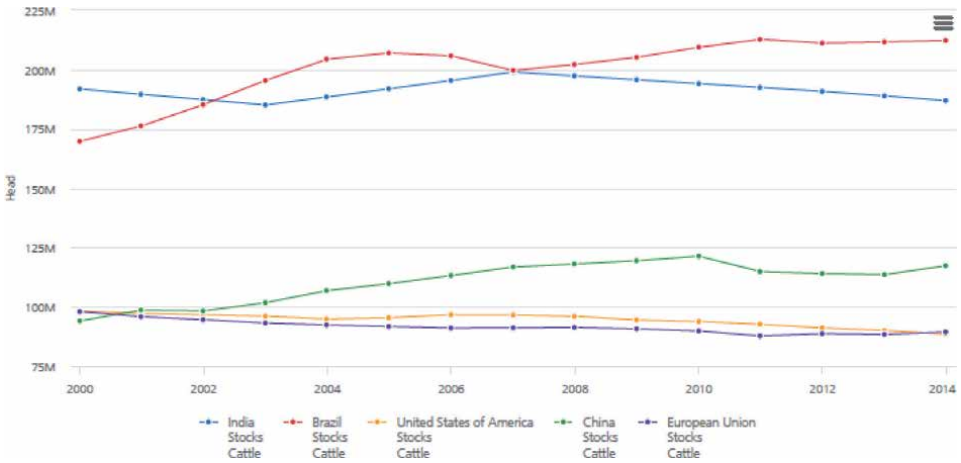

Figure 1.
Countries with the largest cattle herds, 2000–2014, in millions of heads. Source: Prepared by the authors, based on FAO [1].

Agriculture is one of the principal GHG emitting activities in Brazil due to, for example, the methane resulting principally from enteric fermentation and the handling of waste, and nitrous oxide from feces and urine deposited by animals [3]. In the last emissions inventory, agriculture was responsible for around 32% of greenhouse gas emissions in the country, without taking into account the emissions created from the use of energy, measured in the part of energy whose contribution is almost 29%, but which includes a part related to the demand of the agricultural sector for energy and energy resources, as well as emissions from the conversion of forests into pasture, calculated in the part related to changes in land use. Agricultural thus contributes, directly or indirectly, to most greenhouse gases produced in the country, as seen in **Figure 2**.

In relation to the demand for energy, the agricultural sector demanded around 14.08% of the total diesel oil used in the country (approximately 7460,000 m^3) and almost 11.48% of firewood (more than 9000 tons) [4]. In the case of the use of electric energy, the agricultural sector consumed around 5.14% of the final total consumption of electricity in 2015, or 26,870.89 GWh [4].

Despite have a majority renewable energy matrix, as D'Avignon [5] highlights, this does not impede the need for the implementation of complementary renewable energy programs, above all with short-term energy policies, given the environmental limitations on the construction of new hydro-electric power plants with large reservoirs[2], which include the majority of those constructed in Brazil, or the hydric crises which have occurred in recent years, causing serious problems with the supply of electric energy, imposing the need, for example, for rationing and the introduction of non-renewable sources of energy and more sources of GHG emissions, following the insertion of thermoelectric power plants (above all, coal and oil) [6–10]. A way to reduce dependence on hydroelectricity for the generation of electric energy and with a lower environmental impact is a policy of complementarity with new sources of renewable energy generation [5]. One of the possible renewable energy sources is biogas produced through the confinement of cattle.

[2] It should be emphasized the hydroelectricity continues to be the principal source in the Brazilian energy matrix. According to BEN, hydraulic energy was responsible for 64% of the total offer of electric energy in 2015 [6].

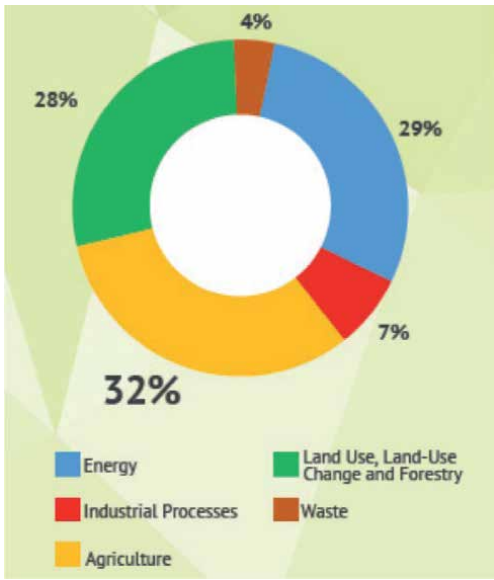

Figure 2.
Participation of each sector in the GEE emissions, in CO_2e, in 2010. Source: [12], p. 18.

2. Biogas produced from cattle confinement as a complementary and strategic energy resource in Brazilian energy policy

According to the FAO [11], it is estimated that livestock emit around 7.1 $GgCO_2eq$ per year or 14.5% of total global emissions. The same study demonstrates that most emissions are related to the production of beef (41%) and milk (20%). Emissions are also linked to the production and processing of animal feed, enteric fermentation, the handling of waste and the remnants of processing, and the transport of products of an animal origin [11]. In the Brazilian case, in the last inventory, emissions related to enteric fermentation accounted for 11,158 $GgCH_4$ or around 66.9% of total methane emissions in the country in 2010 and in the case of animal waste, this value was 3.6% of total methane emissions, 608.1 $GgCH_4$ [12].

Confinement can contribute to the generation of electric energy with lower GHG emissions. This reduction is due to the conversion of methane generated during the beef production process into biogas as a source of energy for the energy sector, slaughterhouses, and abattoirs in particular, part of the highly energy intensive agricultural sector. At the same time, as it is a system with higher production costs in relation to the extensive system[3], biogas also contributes to reducing these costs. The **Figure 3** shows how the confinement rate in Brazil is still very low.

Biogas is a gaseous mix produced by the anaerobic decomposition of organic material whose characteristics and typical composition, according to Persson et al. [14], is around 53–70% methane and 30–47% carbon dioxide, with

[3] Feeding the beef cattle herd in Brazil is based on pasture, the most economic and practical means of feeding cattle. This contribute to the lower costs of beef production which are competitive in relation to the United States, Australia, and various European countries. In the latter, the predominant system is confinement, dependent on other economic factors which oscillate, such as variations in the prices of grains or fossil fuels [3, 7–10, 12, 13]. The generation of biogas help to minimize the costs associated with confinement.

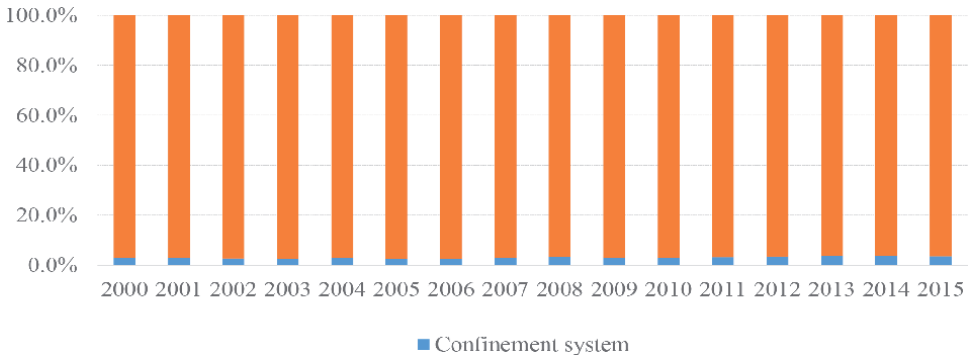

Figure 3.
Participation of cattle in confinement system and cattle in extensive system or rational grazing system on the total Brazilian cattle herd, 2000-2015. Source: Prepared by the authors, based on FNP (2002, 2005, 2012 and 2016).

traces of at least 1000 ppm of sulfuric acid and at least 100 ppm of ammonia. Agricultural waste treatment systems are principally responsible for the use of the anaerobic digestion process [15].

Biogas is widely used in some countries, such as China and India, for cooking and lighting in rural areas. In Brazil, the use of this energy source was intensified in the 1970s, through governmental programs in small rural properties to reduce the dependence on LPG and the negative impacts of raising animals, as well as to increase the income of landowners. However, the lack of technical knowledge to construct and operate bio-digesters was one of the principal problems of the use of biogas in the country [16–18]. Usually, the most common uses of biogas, seen in a wide variety of countries, is its use for heating and the generation of electricity. In the case of the use of biogas for electricity generation, various technologies are available, with the principal applications being gas turbines and internal combustion generators [19].

The generation of biogas from animal waste depends on different factors: temperature, pH, alkalinity, the characteristics of animal waste, and how it is handled in the production system. As a result, animal diet gives the waste distinct potentials to produce gas [16–18]. The production of biogas is possible with the confinement of cattle before slaughter and from effluents from the production process in slaughterhouses and abattoirs.

Two types of confinement are practiced in Brazil [12]. Confinement and semi-confinement during the dry periods of the year, from May/June to October/November. For the confined cattle, the fattening period lasts on average four months, and two for semi-confined. **Table 1** shows the growth of the confinement system in the country in recent years. In 2000, the number was 4.39 million, of which 55.6% were semi-confined and 44.4% confined. In 2015, it reached a little more than 6.7 million, with approximately 40.3% being semi-confined cattle and almost 59.7% confined. In the 2000–2015 period, there was an increase of 34.6% [20–23].

The largest producers of beef in the world, the United States and Australia [24], have an average rate of confined cattle of around 50%, some years some US states, such as Texas, have an even higher rate. In the Brazilian case, this rate was 3.5% in 2016. Few states had rates much higher than the national average, notably São Paulo with 4.8% of the national herd and a confinement rate of 10.4% of its total herd. However, in many states there is little or no confinement though they have, at the same time, a large share of the national herd, as is the case of the state of Pará with approximately 10.1% of the total of the national beef herd in 2015 and

Type of production system	2000	2001	2002	2003	2004	2005	2006	2007
Confinement	1,950,000	1,868,000	1,906,000	2,039,000	2,427,000	2,305,000	2,317,999	2,572,984
Semi-confinement	2,440,000	2,565,000	2,432,000	2,310,000	2,726,000	2,481,000	2,365,160	2,504,000
Total (confinement +semi-confinement)	4,390,000	4,433,000	4,338,000	4,349,000	5,153,000	4,786,000	4,683,159	5,076,984
Type of production system	**2008**	**2009**	**2010**	**2011**	**2012**	**2013**	**2014**	**2015**
Confinement	2,989,008	2,901,734	2,756,201	3,377,311	3,670,401	4,068,628	4,201,734	4,008,764
Semi-confinement	2,804,000	2,533,191	2,583,042	2,564,146	2,653,589	2,730,584	2,800,802	2,702,774
Total (confinement +semi-confinement)	5,793,008	5,434,925	5,339,243	5,941,457	6,323,990	6,799,212	7,002,536	6,711,538

Source: Prepared by the authors, based on FNP [20–23].

Table 1.
Quantity of confined cattle, semi-confined cattle and total cattle herd with some type of confinement in Brazil, 2000–2015.

with less than 1% of the cattle in the state being confined. This panorama shows the possibility of expanding beef cattle confinement in Brazil in comparison with the rates of confinement in some Brazilian states, but above all in various other countries.

The greater economic competitiveness of the extensive pasture system in relation to the intensive one with confinement can be compensated by the increase of beef production and the reduction of costs through the production of a new energy source (biogas), as well as the reduction in the emissions produced using this source of energy, and a lower dependence on electricity by the agricultural sector. Biogas can thus be an interesting and strategically positive energy policy.

3. Methodology

To calculate the potential to generate biogas from waste created during the beef production chain and the reduction of GHG in different scenarios, various hypotheses and parameters were considered based on the ultimate inventory of Brazilian greenhouse gas emissions [12, 25] and the research carried out by D'Avignon [26], as described below.

The slaughter age of animals fed only on pasture is around 36 months[4], and is inversely proportional to the increased intensification of the productive system, being reduced to 18 months with confinement or 26 months with semi-confinement.

In Brazil, the average time of confinement after weaning is 4 months, though it is possible to expand this to six months, in the driest period, between May and October, while for semi-confinement, the average time can be increased by two months, rising to three months.

It is estimated that biogas with 60% methane is generated because of animal waste per day (4.05 m^3/animal/day). This value was calculated based on the methane information emitted per head of cattle, in accordance with the latest national inventory of greenhouse gas emissions [12].

For the generation of biogas from the effluents produced during animal slaughter, the value of 7,4872 m^3/head slaughtered[5] was estimated by D'Avignon [26], for a large part of abattoirs existing in the country in 2010, responsible for approximately 90.8% of cattle slaughtered in the country. In this research the need for electric energy in the productive process was estimated at 39 kWh per head of cattle slaughtered.

Biogas can thus be generated by two distinct sources: the waste produced by the animal and the effluents generated during animal slaughter. However, with the use of bio-digesters, neither are altered in relation to enteric fermentation, irrespective of changes in the productive system. Intensive and extensive systems emit methane, but with a difference in the number of days. The lower the number of days, the lower the emission of methane due to enteric fermentation. Considering all parameters and hypothesis, biogas production potential is given by the following equation:

[4] There are cases in some parts of Brazil with a slaughter age above 36 months, and even reaching 44 months. However, in most of Brazil, with an extensive system, the slaughter age is around 36 months.

[5] This calculation was based on the sum of the potential of biogas with cattle fat and rumen of around 3.45 m/head slaughtered and with blood, approximately 4.04 m/head slaughtered.

$$g_{biogas} = \left[\left(N_{conf} \right) * \left(\text{number of days} \right) * \left(q_{ent\_fer} \right) + \left(N_{ab} \right) * \left(q_{abat} \right) \right] / FC\left(m^3 \right) \quad (1)$$

where:

- g_{biogas} = biogas production potential, in MW.
- $FC(m^3)$ = conversion factor of m^3 to MWh, in which case the value is 0,00043909.
- N_{conf} = number of confined cattle heads.
- q_{ent_fer} = amount of biogas produced through manure management. In the article, the authors propose 4,05 m^3/animal/day.
- number of days = number of days with confined cattle. For example, cattle confined for 4 months, total days of confinement, 120 days.
- N_{ab} = number of slaughtered cattle in the year.
- q_{abat} = amount of biogas produced through abatement. In the study, this value is 7,4872 m^3/slaughtered head.

If the biogas is applied to the generation of electricity, there is an increase in the abatement of emissions through the reduction of the use of non-renewable sources and many GHG emitters, normally used by a large part of the agricultural sector. This potential reduction also should be calculated in relation to the grid. The use is proposed of the emission factors calculated by MCTI [27] and applied in the inventories existing for the 2006–2015 period. To analyze the years between 2000 and 2005, the factor observed in 2006 was applied, and for future scenarios the authors proposed the use of the MCTI study [25] which calculated the factors for 2020, 2025, and 2030, while for 2015–2020, 2020–2025, and 2025–2030, the calculation will be based on the linear tendency in each period, measuring the annual rates of emission factors in the initial and end years. From this the emission factor results are obtained for the national grid, as described in **Table 2**.

In the case of projections for electricity demands, it is proposed to use the average rate calculated by EPE [4] with a growth in energy sources projected until 2050 for the agricultural sector. According to EPE [28], per year, the electricity consumption of the agricultural sector will be almost 47,101,500 MWh[6]. This is equivalent to an increase of around 42.95% in electricity consumption in the 2015–2030 period. Between 2015 and 2030, the annual rate of growth in the consumption of electricity is estimated at 3.81%. These estimations are important to measure how biogas can contribute to meet the growing demand for electricity in the agricultural sector.

[6] In the study made of projections for energy demand, EPE [10], the total consumption of energy (without detailing the type of source) is given in millions of TEP for the year 2030. In the distribution chart for each source, it is shown that the share of electricity will be 27% in 2030. Based on this information and knowing that the conversion of TEP to kWh is 11.63x103, the result is obtained in kWh and afterwards multiplied by 103 to determine the final value in MWh.

Factor emission – national grid - tCO_2/MWh	**2000–2006**	**2007**	**2008**	**2009**	**2010**	**2011**	**2012**	**2013**	**2014**	**2015**	**2016**	**2017**	**2018**
	0.0323	0.0293	0.0484	0.0246	0.0512	0.0292	0.0653	0.0960	0.1355	0.1244	0.0960	0.0740	0.0571
	2019	**2020**	**2021**	**2022**	**2023**	**2024**	**2025**	**2026**	**2027**	**2028**	**2029**	**2030**	
	0.0441	0.0340	0.0421	0.0521	0.0646	0.0799	0.0990	0.1106	0.1235	0.1379	0.1540	0.1720	

Source: Prepared by the authors, based on MCTI [25] and MCTI [27].

Table 2.
Emission factor of the national grid (tCO_2 / MWh) in Brazil (2000–2015).

However, the use of biogas produces GHG emissions, as indicated by Gómez et al. [28]. According to IPCC, the burning of biogas results in CH_4 and N_2O emissions, which are considered by subtracting them from the reduction. The standard values supplied by Gómez et al. [13] of 1 and 0.1 kg GHG/TJ biogas for methane and nitrous oxide, respectively, were used. These values will be considered in the final calculation of total emissions in each of the scenarios proposed in the article.

In the case of emissions avoided using biogas, the result is the difference between the emissions generated during the beef production process, irrespective of the system, discounting the emissions avoided using biogas as electricity. Then, emissions with the use of biogas are given by the following:

$$E_{biogas} = \left(g_{biogas} * FE_{CH4} * FC(TJ)\right)/1000 + \left(g_{biogas} * FE_{N2O} * FC(TJ)\right) \quad (2)$$

where:
E_{biogas} = biogas burning emission, in tCO_2e,
g_{biogas} = biogas generation potential, in MW.
FE_{CH4} = methane emission factor with the use of biogas (1 kg/TJ).
FC(TJ) = conversion factor from TJ to MWh (0,003599712).
FE_{N2O} = nitrous oxide emission factor with the use of biogas (0,10 kg/TJ).
For emissions abated with biogas, the equation is represented by:

$$E_{abat}^{biogas} = g_{biogas} * f_{grid} \quad (3)$$

where:
E_{abat}^{biogas} = emission abated using biogas in tCO_2e,
g_{biogas} = biogas generation potential, in MW.
f_{grid} = grid emission factor, in tCO_2/MWh (see **Table 2**).
So, the final abated emissions (EF_{biogas}) using biogas are given in the formula:

$$EF_{biogas} = E_{abat}^{biogas} - E_{biogas} \quad (4)$$

To analyze the impact of increased confinement times and rates due to the use of biogas for the generation of energy for the agricultural sector and the reduction in GHG emissions, three different intensification scenarios are proposed, in addition to the reference one. One with the increased confinement time, another with the elevated confinement rate, and finally joining all the scenarios for the intensification of livestock raising through confinement.

4. Scenarios

4.1 Reference scenario

In this scenario, from 2016 until 2030, an average growth rate equal to all the variables seen in the 2000–2015 period is proposed.

In the reference scenario, confinement rates will follow the linear growth tendency observed between 2000 and 2015. In other words, semi-confined cattle will grow at an average annual rate of 0.35%, equal to the growth observed in the

period, 9.7%, and confined cattle at an average annual rate of 4,9%, with a growth of around 51.4% occurring during the period. At the same time, the same hypothesis of linear growth of confinement will be applied to both the size of the beef herd and the number of animals slaughtered in the country. In the case of the beef herd, during this period there was an increase of around 1.6% per year (21.4% in the period as a whole) and in the number of slaughtered animals a little less than 1% per year (13.4% between 2000 and 2015).

As **Table 3** shows, the number of heads of cattle confined will rise from a little over four million, in 2015, to just over 8.2 million in 2030. By 2030, the number of semi-confined heads of cattle will be a little over 2.8 million, while in 2015, there were around 2.7 million. If we compare the number of heads with some type of confinement in relation to the total heads of the beef herd, the more intensive beef production system rises from almost 3.5%, in 2015, to 4.5%, in 2030. Finally, the total cattle herd, without dairy cows, will rise from almost 193.5 million, in 2015, to a little more than 246.2 million in 2030.

In relation to slaughter, the values range from a little above 41 million head of cattle in 2015, to around 47.4 in 2030. The demand for energy for the beef slaughter process will rise from 1600.3 GWh to around 1847.1 GWh (**Table 3**).

4.2 Scenario 1: increase in confinement time

From 2016 until 2030, in relation to the reference scenario, in scenario 1 confinement time increases from 4 to 6 months, and from 2 to 3 months for semi-confinement. The only change in relation to the reference scenario is the confinement time.

The increase in confinement does not result in changes to the initial values calculated for the reference scenario. For this reason, the values of numbers of heads of confined, semi-confined, slaughtered, and total cattle, demand for energy remain the same, as described in **Table 3**. This parameter will alter the potential of the generation of biogas, as will be observed in the results, and consequently the emissions presented reduced somewhat the total emissions generated by animal slaughter over the years, with these small changes in emissions being observed in relation to the reference scenario in the results present in the next item.

4.3 Scenario 2: increase in the confinement rate

In the period 2016–2030, in scenario 2 there is an increase in the rate of cattle with some type of confinement from the rate seen in 2000–2015, rising from 3.5% to 10.9% by 2030, an annual rise of around 9.7%. This hypothesis will allow the rate of cattle with some type of confinement to reach by 2050 the minimum rate practiced in some countries, such as Australia, or as seen in the state of Texas in the US, of approximately 50% in relation to the total beef herd.

With this, as shows, the value of confined and semi-confined cattle will be altered, and the others maintained in accordance with the calculations made for the reference scenario. Since the confinement rate does not indicate the type of confinement, the hypothesis used to calculate the number of confined and semi-confined cattle from 2015 onwards was the average rate of confined and semi-confined cattle in relation to the total cattle with some type of confinement between 2012 and 2015. The reason for this hypothesis is the tendency for these rates to stabilize in recent years, with little variation, as seen in **Figure 4**.

Between 2012 and 2015, the average of this rate for confined cattle was around 59.4%, with an oscillation lower than 2%, also seen in semi-confined cattle.

Baseline scenario/Scenario 1							
	2000	**2005**	**2010**	**2015**	**2020**	**2025**	**2030**
Confinement (cattle heads)	1,950,000	2,305,000	2,756,201	4,008,764	5,097,248	6,481,284	8,241,122
Semi-confinement (cattle heads)	2,440,000	2,481,000	2,583,042	2,702,774	2,750,324	2,798,710	2,847,948
Cattle herd (cattle heads)	151,990,505	186,530,771	186,616,195	193,448,415	209,643,506	227,194,417	246,214,652
Abatement (cattle heads)	35,550,700	44,319,921	40,848,429	41,033,569	43,043,047	45,150,932	47,362,043
Energy demand (MWh)	1,386,477.3	1,728,476.9	1,593,088.7	1,600,309.2	1,678,678.8	1,760,886.3	1,847,119.7

Source: Prepared by the authors, based on FNP [20–23] and D'Avignon [26].

Table 3.
Number of confined heads, semi-confined heads, total heads and slaughtered heads, and energy demand (MWh) for the baseline scenario and scenario 1, in Brazil, in 2000, 2005, 2010, 2015, 2020, 2025 and 2030.

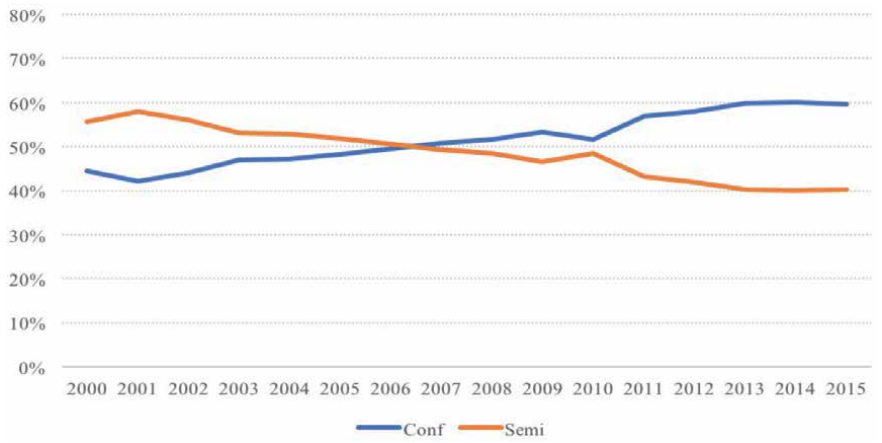

Figure 4.
Rate of number of confined cattle on total cattle with some type of confinement and number of semi-confined cattle on total cattle with some confinement in Brazil, % (2000–2015). Source: Prepared by the authors.

For semi-confined cattle, this average rate was 40.6%. Therefore, the number of confined cattle will rise from a little over four million in 2015 to around 15.9 million in 2030. Semi-confined cattle will increase from almost 2.7 million (2015) to almost 10.9 million. Total cattle with some type of confinement in 2030 will be around 26.8 million, representing an increase of 74.2% in relation to 2015.

4.4 Scenario 3: increases in the rate of cattle with some form of confinement and the time of confinement

Joining scenarios 1 and 2 creates scenario 3, thus, as has been seen in the previous scenarios, only the basic values for the calculation of the creation of biogas and emissions are altered. Permitted in this scenario is the analysis of all the proposed possibilities for the intensification of livestock raising through the confinement of cattle, and through the increase in both confinement time and the total rate of cattle in some form of confinement.

After the analysis of each of the proposed scenarios and how the basic variables are modified in the three scenarios, the biogas generation potential and to what extent this can meet the demand for energy of slaughterhouses and abattoirs, and the agricultural sector, is examined, as well as the impact on emissions of the insertion of biogas in the energy matrix. In short, it shows how cattle confinement can contribute to energy policy as a complementary renewable source capable of guaranteeing energy security from the point of view of supply without harming the environment.

5. Results

There is a high potential for the use of biogas. Even without any intensification policy it will be possible to meet almost 80% of the energy demands of abattoirs and slaughterhouses in 2015. In 2030, not only will it be possible to meet all energy demands necessary for animal slaughterhouses, but also energy can be offered to the Brazilian electricity system with an addition of around 371.1 GW/h year. In the case of the agricultural sector, first, it is estimated that the demand for electricity of slaughterhouses and abattoirs will represent almost 6% of the total electricity consumed by the sector in 2015. By 2030 it is estimated that this value will be approximately 4%. The insertion of biogas as a source of electricity can be noted that renewable resources

can meet approximately 14.9% of the electricity demands of the agricultural sector in 2030, in a scenario with both an increase in the confinement rate and an expansion of time spent in confinement (Scenario 3). The importance of the use of biogas as an alternative energy source is so relevant that without any alteration in the country's beef productive system in 2015 biogas could generate sufficient electricity to meet about 4.8% of the demands of the agricultural sector, and in 2030 this value would be practically the same, a little more than 4.7% (**Figure 5**). Changes in confinement time or in the confinement rate can increase the offer of biogas and can increase even more the additional offer to the grid with an addition of almost 1402.4 GW/h (scenario 1), 2867.1 GW/h (scenario 2), or 5146.3 GW/h (scenario 3). As **Figure 6** shows, it is possible to create more than three times the energy demand necessary for animal slaughter (offer of biogas/energy demand = 378.6%) in 2030.

It can be noted that increased confinement time (scenario 1) creates a lower increase in the offer of biogas for electric energy when compared to the increase

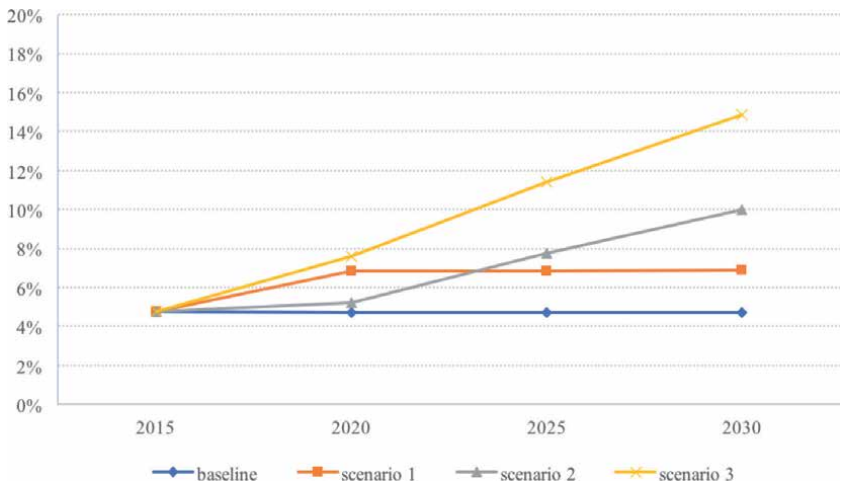

Figure 5.
Relation between the supply of biogas and electricity demand for agricultural sector, for each scenario, % (2015, 2010, 2025 and 2030). Source: Prepared by the authors.

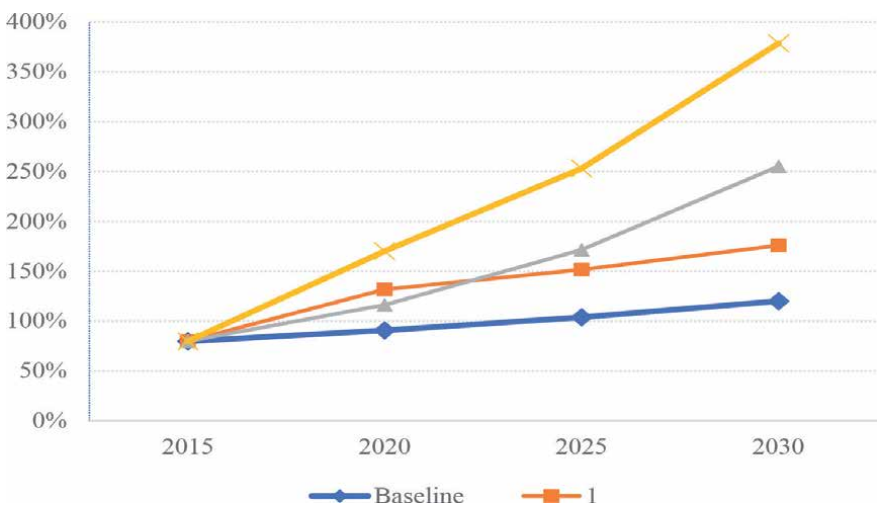

Figure 6.
Relation between the supply of biogas and the electricity demand for slaughterhouses, % (2015, 2020, 2025 and 2030). Source: Prepared by the authors.

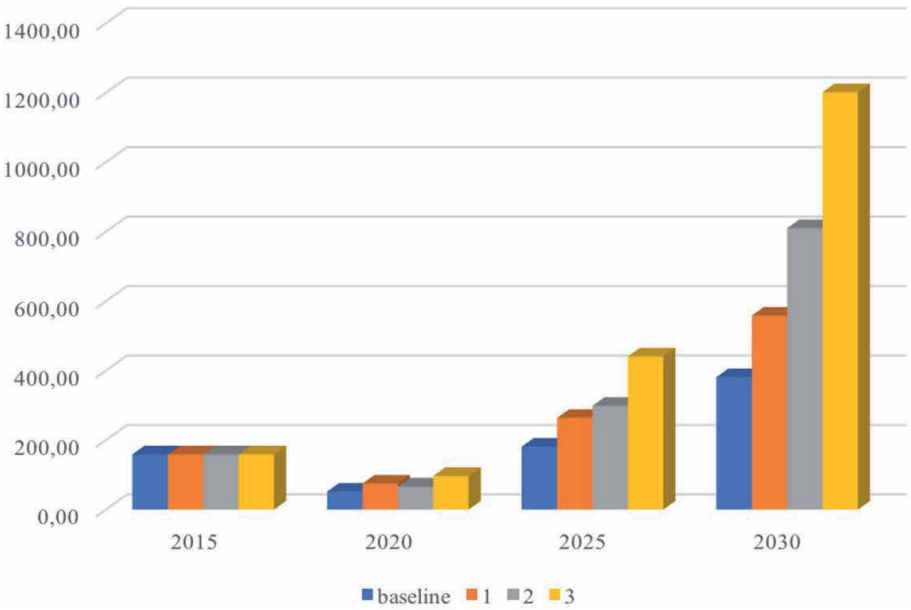

Figure 7.
Emission reduction with biogas use, for each scenario, in $GgCO_2e$ (2015, 2020, 2025 and 2030). Source: Prepared by the authors.

in the confinement rate and, above all, that the sum of the different intensification polices results in a significant increase. Thus, this result shows the strategic importance of biogas in Brazil as an energy source capable not only of meeting the demand of slaughterhouses and abattoirs, but also part of the energy demands of the agricultural sector.

An additional positive effect of the use of biogas, in addition to the expansion of energy demands, is on the reduction of GHG emissions. Despite having a low grid emission factor due to the high share of hydroelectricity in the Brazilian energetic matrix, the insertion of a renewable source with the low GHG emissions helps keep the emissions of the Brazilian electricity sector low. In the scenario with the implementation of all intensification policies in the agricultural sector (Scenario 3), it is possible to reduce in the long term (2030) a little more than 1201.56 $GgCO_2e$. Without any policy, this reduction will reach 381.12 $GgCO_2e$, as **Figure 7** shows.

6. Conclusion

Despite being one of the largest beef producers in the world, Brazil still has low productivity indices in the livestock sector, in which the predominance of extensive system means that the country has a stocking rate of between 1 and 2 heads of cattle per hectare. Given this fact, the country has great potential to intensify the sector with the possibility of the reduction of the demand for electricity in abattoirs and slaughterhouses using biogas and even the agricultural sector as a whole and to reduce somewhat greenhouse gas emissions generated by the sector through the introduction of a new renewable and cleaner energy source.

In the scenarios analyzed, it was perceived that the use of biogas in beef cattle confinement is an interesting complementary energy policy, which fits into the need to diversify renewable energy sources to reduce potential problems in electricity supply, in relation to an energy matrix that is dependent on hydroelectricity to

meet the growing demand of the agricultural sector for electricity. In scenario 3, it can be observed that the application of policies to intensify the livestock sector allows a considerable reduction in GHG emissions and to meet the needs of both the agricultural sector and the totality of the energy demand of slaughterhouses and abattoirs, which are close to the herd, facilitating the use of biogas to supply them.

More profound studies, assessing the economic question, such as the costs of expanded confinement and if, from the economic point of view, intensification is attractive, need to carry out to confirm whether, from the technical point of view, increasing beef confinement is an interesting energy policy to increase the offer of electricity and to reinforce the energy security of Brazil. The scenarios show the need to modernize livestock raising through the better use of the waste and the effluents created during the slaughter process and to propose as a short and mid-term energy policy.

Therefore, the conclusion is reached that the use of biogas is important to reduce greenhouse gas emissions, contribute to cleaner energy generation and reduce the energy dependence of the agricultural sector in Brazil. In the face of climate change, this energy resource can help the country to help meet the goals proposed under the Paris Agreement and attract foreign investment in view of the enormous potential to generate energy and reduce emissions.

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Section 3

Health Perspective

Chapter 5

Serological Monitoring for *Leptospira* Spp. and Monitoring of Productive and Reproductive Indices on Dairy Farm

Leandro Temer Jamas, Rodrigo Rhoden Barcellos, Carlos Roberto Padovani, Cassiano Victória and Helio Langoni

Abstract

Leptospirosis is a zoonosis caused by spirochetes of the genus *Leptospira*. It has a worldwide distribution with greater occurrence in tropical and subtropical countries. It is endemic in Brazil. It affects domestic, wild and production animals. The goal of this study was to assess dairy herd productive and reproductive indexes on a monthly basis by serologically monitoring the infection dynamics on two experimental groups: one with animals with negative results at study onset (G-1) and another with animals tested positive for at least one leptospira serovar (G-2). The serum microscopic agglutination test (MAT) was employed. Animals with titer equal to or greater than 100 IU were considered reactive. Animals were evaluated for productive and reproductive indexes based on data provided by the dairy's IT system. Blood was collected from all animals in both groups once a month for nine months. Analysis showed interference between animals seroreactive to leptospirosis and both milk production and number of pregnancies for G-2 at collection moments 3, 4, 5, 6, 7 and 9 whereas for G-1 the same indexes showed decrease only in the 5th and 9th study months. The most prevalent serovars were Hardjoprajitino 59.5%, Pyrogenes 21.04%, Pomona 11.07%, Wollfi 11.07%, Hardjo 8.78%, Guaricura 6.55%, Copenhageni 5.09%, Icterohaemorrhagiae 1.11%, and Ctg 0.83%. Serovar Hardjoprajitino showed a relationship with herd milk production decrease.

Keywords: leptospirosis, animal production, dairy cattle

1. Introduction

Leptospirosis is a worldwide bacterial zoonosis showing greater occurrence in tropical and subtropical countries. It is transmitted mainly through direct contact with animals or urine but can also be acquired indirectly by ingesting contaminated water or food. The disease is typically occupational affecting particularly farmers, slaughterhouse workers, veterinarians and their co-workers [1].

Leptospirosis brings economic loss to cattle raisers as it causes reproductive disturbances like abortion or infertility. It is considered a reproductive system disease [2].

Clinical signs can be chronic such as abortion, mainly at the pregnancy's middle third, around the fifth month, estrus repetition and stillbirths as well as placental retention not always occuring. It can cause agalactia or decrease in milk production as well as infection of young calves [3].

Cattle are the main reservoirs of serovars Hardjo [4], and others such as Pomona and Grippotyphosa [5]. They are the preferential hosts of serovar Hardjo. Serovar Hardjoprajitino is responsible for decreases in cattle milk production and conception rates [6], and serovar Hardjobovis is associated with reproductive failures [2–7].

The main serovars found in Brazil are Hardjo, Wolffi, Pomona, Grippotyphosa, Icterohaemorrhagiae [3]. There is a prevalence of serovar Hardjoprajitino, also present in commercial vaccines. However, as with other domestic mammals, cattle can be infected by any pathogenic serovar [8]. Despite some degree of agent species selectivity, the disease is not serovar-specific.

Considering the impact of leptospirosis on cattle breeding as well as its effects on human and animal health, the present study was proposed with the goal of evaluating the consequences of leptospiral infection on the pregnancy and milk production rates of a confined dairy cattle herd with respect to the serological response to 16 serovars of Leptospira spp., of importance for herbivores, during 9 months, having it associated with productive, referring to milk production, and reproductive, referring to pregnancy rate, indexes as well as monitoring leptospiral infection evolution in two groups set up and kept under similar conditions, one with animals serologically positive for at least one of the evaluated serovars and another, the control group, with animals serologically negative at study onset, the results thereof compared vis-à-vis the studied variables.

2. Material and methods

With owner's consent secured, the study took place in a dairy property the authors were familiar with. These premises were selected due to the permanent availability of veterinary assistance and the authors' good understanding of its zoosanitary practices. The dairy is a fenced property capable of animal self-replacement, located in the central region of the State of São Paulo, Brazil. Its stock counts 750 animals of which about 400 are of high genetic lineage, pure origin Black and White Dutch lactating cows kept in a semi-confinement system and milked three times a day. The production system is completely computerized, allowing data to be obtained on a monthly basis to evaluate the individual and herd productive and reproductive indexes. Milking is carried out with the help of a carousel-type parlor.

Animals are vaccinated against IBR, BVD, brucellosis and leptospirosis one week before dry-off, approximately 60 days before parturition, and receive a second shot 30 days later. Lactating cows are vaccinated between 120 to 128, 270 to 278 and 420 to 428 days of the lactation cycle.

The experimental groups were formed spliting 202 lactating animals in two groups. One group had 50 animals with non-reactive results to anti-leptospiral antibodies (G-1) while the other has 50 sera reactive animals with a microscopic agglutination test (MAT) titer ≥100 IU for at least one Leptospira serovar (G-2). G-2 was reduced to 39 animals by the end of the study as 11 were discarded by the owner during the experimental period. Both groups were set up with animals picked at the beginning of their lactation cycles affording longer monitoring times within their milk production periods.

G-1 and G-2 Blood samples were collected monthly for 9 months by mammary vein puncture to assess herd infection dynamics. In order to diagnose infection,

MAT were performed employing live antigens from 16 serovars belonging to 10 serogroups: serovar Bratislava (serogroup Australis); Castellonis (Ballum); Canicola (Canicola); Djasiman (Djasiman); Grippotyphosa (Grippotyphosa); Copenhageni, Icterohaemorrhagiae (Icterohaemorrhagiae); Pomona (Pomona); Pyrogenes (Pyrogenes); Tarassovi (Shermani); Guaricura, Hardjobovis, Hardjo CTG, Hardjoprajitono, Mini, Wolffi (Sejroe). The titer cut-off point was 100 IU.

Production indexes, such as the monthly average of milk production in liters, as well as reproductive factors such as interval between birth and conception, conception rate at the first service, conception rate in all services, services per conception, age at first delivery and number of lactations were evaluated from each animal's history obtained from the dairy's database.

The results of this longitudinal observational study were analyzed by evaluating the relationship between infection by *Leptospira* spp. and the productive and reproductive parameters of cows. Regarding milk production, every month the corresponding milk output log10 was computed and used first to compute the area under the curve (AUC) for each animal and then the mean and standard deviation.

MAT serovar positivity at the diverse moments was established by means of descriptive statistics where positivity percentages represented the frequency distribution of occurrences. Association of pregnancy to reactive serovar for the two groups in the different moments was carried out with the Goodman association test for contrast between and within multinominial populations [8, 9], whose significance was designated with the help of lowercase and uppercase Latin letters. With significance indicated by lowercase and uppercase Latin letters. Milk production comparison for reacting serovar for each moment was done by independent samples Student's t-test [10]. Statistical results were discussed at the 5% significance level.

3. Results

In 238 group independent blood samples (67.42%) responded to only one serovar with Hardjoprajitino prevalence. In 29 samples (8.51%) Pomona, in 41 (11.6%) Pyrogenes and Wolffi in 18 (5.09%). When two serovars were found, there was again a predominance of the Harjoprajitino serovar in 96 samples (41.9%), Pyrogenes in 53 (23.1%), Pomona in 20 (8.73%), Wolffi in 16 (6.98%), Hardjobovis in 18 (7.8%) and Guaricura in 13 (5.67%). For three serovars, Hardjoprajitino predominated in 25 samples (25.7%), Pyrogenes in 21 (21.6%), Wolffi in 15 (15.40%), Pomona in 12 (12.37%), Copenhageni in 9 (9.27%), Guaricura in 6 (6.18%) and Hardjobovis in 5 (5.15%). For four serovars, Hardjoprajitino was again prevalent in 18 (22.7%) of the samples, Pyrogenes in 15 (18.9%), Pomona and Wolffi in 9 each (11.3%), Copenhageni in 11 (13.9%), Hardjobovis in 7 (8.86%), Guaricura in 5 (6.32%) and CTG in 3 with (3.79%). For five serovars, Hardjoprajitino predominated in 3 samples (25%), each of Pyrogenes, Copenhageni and Pomona in 2 (16.6%), each of Wolffi, Hardjobovis and Guaricura in 1 sample (8.3%). The most frequent serovars were Hardjoprajitino, Pyrogenes, Pomona and Wolffi. These were also prevalent as co-agglutinants for serovars Copenhageni, Guaricura and Hadjobovis with respect to the same serogroup. The large number of serovars with cross-reactions or co-agglutination is noteworthy.

Figure 1 refers to the 39 animals seroreactive at the first collection (G-2) which remained in the study and shared the same environment with those in G-1.

Figure 2 summarizes the pregnancy results and the positive percentage of serovars at each moment, also in G-2. Pregnancy rates decreases can be observed at moment 3 with 76.9%, moment 4 with 74.3%, moment 5 with 76.9% and moment 8 with 79.4% of pregnancy.

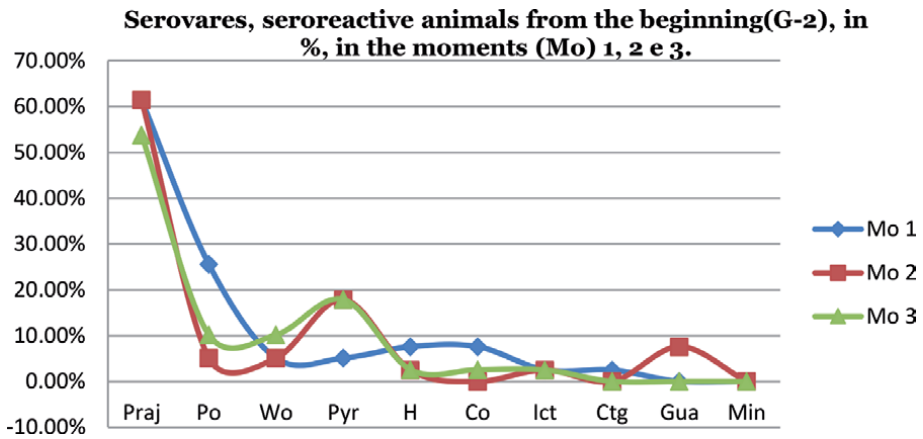

Figure 1.
Distribution of results at the beginning of the study (moments 1, 2 and 3) for the G-2 group, according to milk production and pregnancy. Praj = Hardjoprajitino, Po = Pomona, wo = Wolffi, Pyr = Pyrogenes, H=Hardjo, Co = Copenhageni, Ict = Icterohaemorrhagiae, Ctg = Ctg, Gua = Guaricura, min = mini.

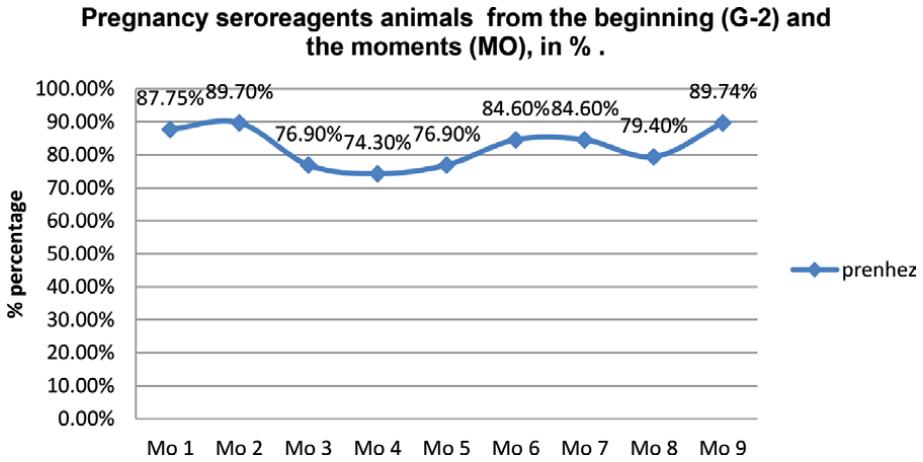

Figure 2.
Dynamics of the result of pregnancy rates in the G-2 group, at times (Mo 1) to (Mo 9). G-2 milk production in liters of milk can be seen for different moments in **Figure 3**.

Table 1 summarizes the results respective of the number of animals, pregnancy in percentage and production in liters of milk at time 2 in G-2.

Serovar Hardjoprajitino was detected at the moment 6 in 18 animals (46.1%), Pyrogenes in 13 (33.3%), Pomona in 9 animals (23%) and Hardjo in 6 animals (15.3%), when decreases in milk production and fertility were observed for infection by the Hardjo serovar, G-2.

Figure 4 shows a serovar Hardjoprajitino participation of 69.2% in moment 8, 64.1% in moment 9 and 41% in moment 7, all in G-2. Variation among seroprevalence percentages at those moments can be seen for the various serovars.

Moment 9 (**Figure 4**) had a higher seroprevalence of serovars Hardjoprajitino in 25 animals (64.1%), Pyrogenes in 17 (43.5%), Guaricura in 7 (17.9%), Wolffi in 4 (10.2%), Copenhageni in 3 (7.6%), Pomona in 2 (5.1%), Hardjo in 1 (2.5%) in G-2.

Figure 5 shows a decrease of pregnancy rates in G-1 at moment 5 which may be related to positivity for serovar Hardjoprajitino. On the other hand, **Figure 6** illustrates G-1 milk production at different times, showing a decrease in milk production at moments 5, 7, 8 and 9 which may be related to calving times. As a matter

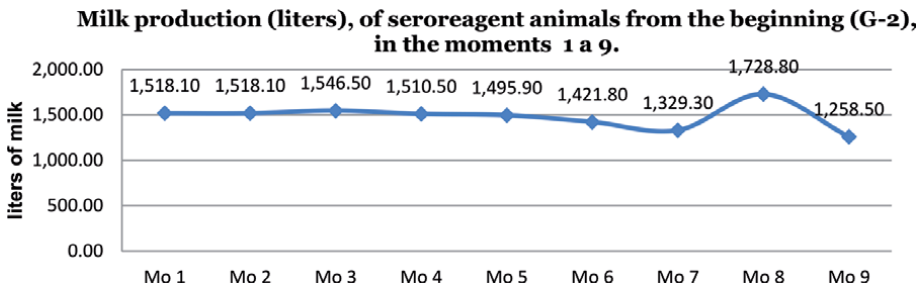

Figure 3.
Dynamics of milk production in liters, in the group of seroreagent animals (G-2), from Mo 1 to Mo 9.

Seroreagents	**Animals**	**(Mo 2)**
Serovars	**N°**	**%**
Hardjoprajitino	24	61,5
Pyrogenes	7	17,9
Wollfi	2	5,1
Pomona	2	5,1
Icterohamorrhagiae	1	2,5
Hardjo	1	2,5
Guaricura	3	7,6
Prenhez em %	32	82
Production in liters of milk	39	1.508,3

Table 1.
Productivity of seroreagent animals (G-2), pregnancy and production in liters of milk, at time 2. Results expressed as a percentage.

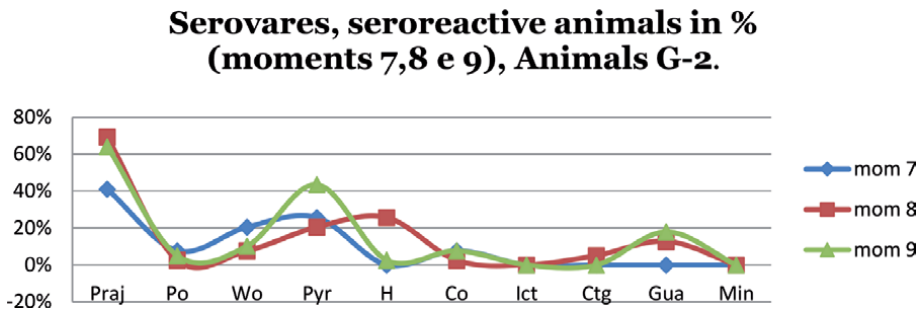

Figure 4.
Kinetics (dynamics) of G-2 antibody titers expression, compared to moments 7, 8 and 9 of observation. Praj = Hardjoprajitino, Po = Pomona, wo = Wolffi, Pyr = Pyrogenes, H = Hardjo, Co = Copenhageni, Ict = Icterohaemorrhagiae, Ctg = Ctg, Gua = Guaricura, min = mini.

of fact, despite attempts to start the experiment with groups as homogeneous as possible, delivery times varied and some animals possibly found themselves in more advanced lactation stages of lactation thus interfering with the group's overall production.

Table 2 shows all the serovars as percentages, at moment 2 for G-1. The pregnancy rate was 87.7% and the milk production 1,963.8 liters. Comparing these

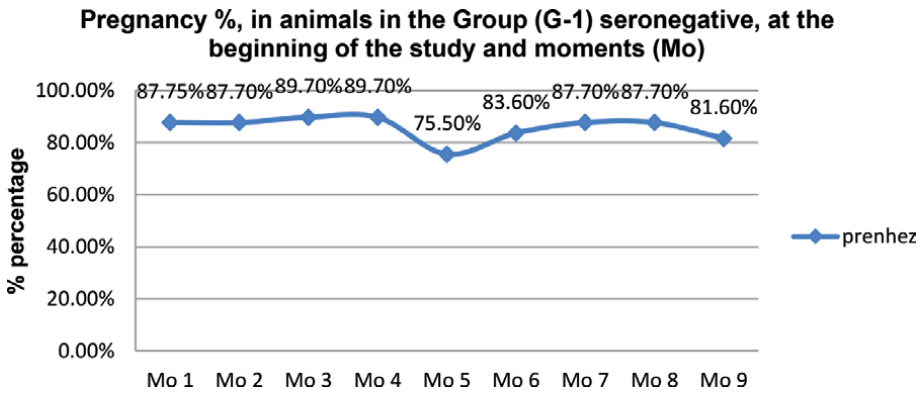

Figure 5.
Dynamics of the pregnancy rate in the G-1 group, from the initial moment Mo 1 to Mo 9.

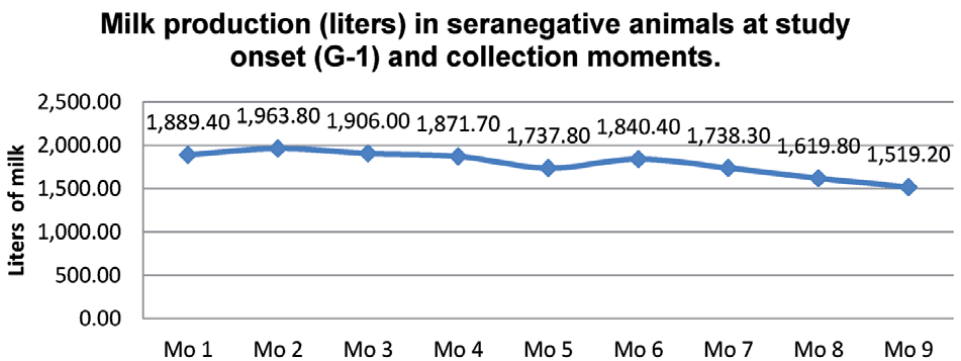

Figure 6.
Dynamics of milk production in liters in the G-1 group, from the initial Mo to Mo 9.

Sororeagents	**Animals**	**(Mo 2)**
Serovars	**N°**	**%**
Hardjoprajitino	18	36,7
Pyrogenes	4	8,16
Hardjo	1	2,04
Wolffi	1	2,04
Ctg	1	2,04
Icterohaemorrhagiae	1	2,04
Prenhez em %	43	87,7
Produção em litros de leite	49	1.963,8

Table 2.
Productivity of seroreagent animals (G-1), pregnancy and production in liters of milk, at time 2. Results expressed as a percentage.

pregnancy rates and milk production with those for G-2, both pregnancy rate and milk production is higher for G-1 at the study onset probably due to lower infection rates of serovars such as Hardjoprajitino in G-1.

At moment 3, G-1, serovar Hardjoprajitino was found in 21 animals (42.8%), Pyrogenes in 12 (24.4%), Pomona in 9 (18.3%), Wolffi in 7 (14.2%), Copenhageni

in 5 (10.2%) and Icterohaemorrhagiae in 2 (4.08%) with a pregnancy rate of 89.7% and milk production of 1,906.8 liters. Hardjoprajitino remained the most frequent serovar with a slight increase when compared to Mo 2. As mentioned for Mo 2, pregnancy rates and milk production were also higher when compared to the earlier moments, for G-2, where serological response with variable antibody titers for one or more leptospiral serovars were present at study onset. Such observation reinforces the importance especially of the serovar Hardjoprajitino to the productive and reproductive aspects of dairy cattle, the focus of the present study.

Figure 7 illustrates the dynamics of antibodies titres regarding the Hardjoprajitino serovar with 59.1%, 55.1% and 46.9% positivity thus confirming the relevance of this serovar for cattle. The participation of serovar Pyrogenes among the serovars that stand out at different times is noteworthy.

At moment 4 for G-1, serovar Hardjoprajitino was obtained in 29 animals (59.1%), Pyrogenes in 5 (10.2%), Pomona in 4 (8.16%), Hardjo in 4 (8.16%), Wolffi in 3 (6.12%), Guaricura in 2 (4.08%), Copenhageni in 2 (4.08%), Castellonis in 1 (2.04%). The pregnancy rate was 89.7%, and milk production 1,872 liters of milk. There was a slight decrease in milk production but the pregnancy rate was the same as for the previous month (Mo 3).

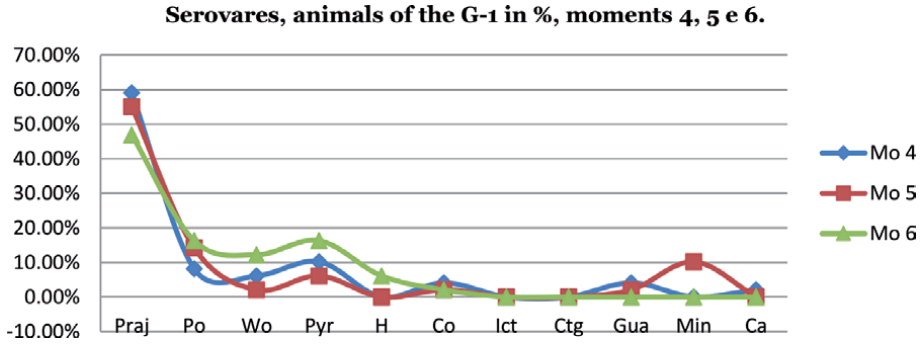

Figure 7.
Kinetics (dynamics) of G-1 antibody titer expression at moments 4, 5 and 6 of observation. Praj = Hardjoprajitino, Po = Pomona, wo = Wolffi, Pyr = Pyrogenes, H = Hardjo, Co = Copenhageni, Ict = Icterohaemorrhagiae, Ctg = Ctg, Gua = Guaricura, min = mini, Ca = Castellonis.

Sororeagents	**Animals**	**(Mo 5)**
Serovars	**N°**	**%**
Hardjoprajitino	27	55,1
Pomona	7	14,2
Mini	5	10,2
Pyrogenes	3	6,12
Guaricura	1	2,04
Wolffi	1	2,04
Copenhageni	1	2,04
Prenhez em %	37	75,5
Produção em litros de leite	49	1.737,8

Table 3.
Productivity of seroreagent animals (G-1), pregnancy and production in liters of milk, at time 5. Results expressed as a percentage.

Table 3 summarizes the G-1 results for moment 5. The pregnancy rate was 75.5%, and milk production volume 1,738 liters. There was a decrease in productive and reproductive indexes with a decrease in pregnancy rate and milk production when compared to previous moments.

Reagent serovars in G-1, moment 6, were Hardjoprajitino in 23 animals (46.9%), Pyrogenes in 8 (16.3%), Pomona in 8 (16.3%), Wolffi in 6 (12.24%), Hardjo in 3 (6.12%), and Copenhageni in 1 (2.04%), with a pregnancy rate of 83.6% and a milk productionn volume of 1,840 liters of milk. There was a decrease in the seroprevalence of Hardjoprajitino from 55.1% to 46.9% and consequently increases in pregnancy rate from 75.5% to 83.6% and in milk production from 1,737.8 to 1,840 liters of milk.

Figure 8 illustrates the dynamics of the response to serovars, in Mo 7, 8 and 9. There are differences among the various moments, seroprevalence oscillating, which is possible since the animals shared the same environment exposing themselves to animal-maintained and environment serovars.

At moment 7 in G-1, serovar Hardjoprajitino can be observed in 7 animals (14.2%), Pyrogenes in 8 (16.3%), Wolffi in 6 (12.2%), Copenhageni in 3 (6.12%), Pomona in 1 (2.04%), with a pregnancy rate of 87.7%, and milk production of 1,738 liters of milk. Despite the lower response to serovar Hardjoprajitino, there was an increase in the pregnancy rate but a decrease in milk production in comparison to moment 6. At this moment there was no response to serovar Hadjobovis. For this same group, in moment 8, serovar Hardjoprajitino was detected in 22 (44.8%), Pyrogenes in 9 (18.3%), both Guaricura and Hardjo in 6 (12.24%), Ctg in 5 (10.2%), Wolffi, Pomona and Icterohaemorrhagiae in 1 (2.04%) each. The pregnancy rate was 89.7% and milk production volume 1,620 liters of milk. There was an increase in the response to serovar Hardjoprajitino, from 16.3% to 44.8% and the serovar Hardjobovis, not found at moment 7, appears in 12.24% which must have influenced the decrease in milk production from 1,738 to 1,620 liters, despite pregnancy rate showing a slight increase from 87.79% to 89.7%.

Table 4 summarizes the most frequent serovars for moment 9, with a pregnancy rate of 81.6% and a milk production volume of 1,519 liters. A decrease in the response to serovar Hardjoprajitino as well as a lack of response to serovar Hardjobovis were observed. There was also a decrease in pregnancy rate and milk production despite the lower response to the serovar Hadjoprajitino, a reduction from 44.8% to 24.4% at moments 8 and 9, and the non-response to serovar Hadjobovis at that moment. Regarding the decrease in milk production also seen in G-1, it should be noted that many animals might have been in an advanced stage,

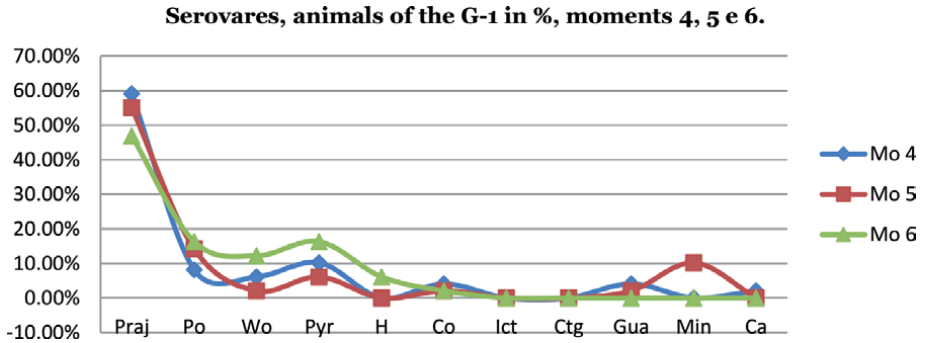

Figure 8.
Kinetics (dynamics) of G-1 antibody titer expression at moments 7, 8 and 9 of observation.
Praj = Hardjoprajitino, Po = Pomona, wo = Wolffi, Pyr = Pyrogenes, H = Hardjo, Co = Copenhageni, Ict = Icterohaemorrhagiae, Ctg = Ctg, Gua = Guaricura, min = mini, Ca = Castellonis.

Seroreagents	**Animals**	**(Mo 9)**
Serovars	**N°**	**%**
Hardjoprajitino	12	24,4
Pyrogenes	17	34,6
Guaricura	4	8,16
Pomona	5	10,2
Copenhageni	2	4,08
Prenhez em %	40	81,6
Produção em litros de leite	12	1.519

Table 4.
Productivity of seroreagent animals (G-1), pregnancy and production in liters of milk, at the moment 9. Results expressed as a percentage.

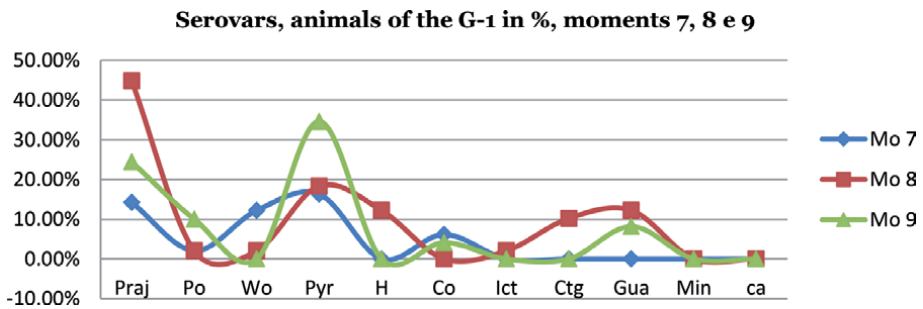

Figure 9.
Average percentage of animals in the G-1, at moments 1 to 9 of observation. Praj = Hardjoprajitino, Pyr = Pyrogenes, Po = Pomona, wo = Wolffi, H = Hardjo, Co = Copenhageni, Ict = Icterohaemorrhagiae, Ctg = Ctg.

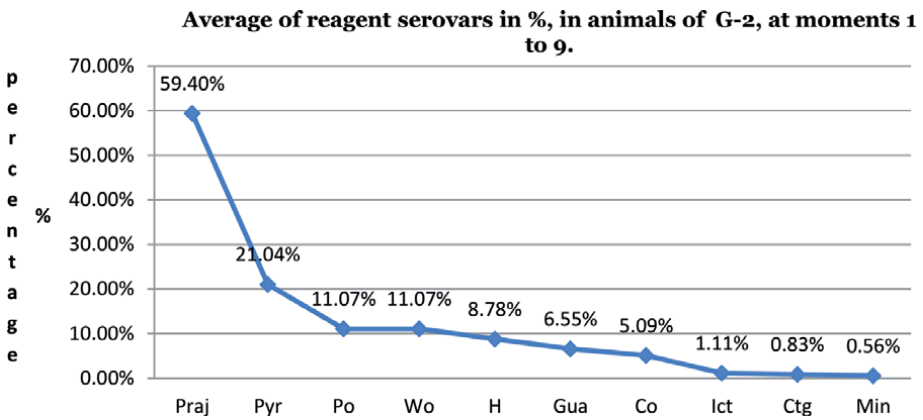

Figure 10.
Average percentage of animals in the G-2, at moments 1 to 9 of observation. Praj = Hardjoprajitino, Pyr = Pyrogenes, Po = Pomona, wo = Wolffi, H = Hardjo, Co = Copenhageni, Ict = Icterohaemorrhagiae, Ctg = Ctg.

near the end of lactation with a consequential decrease in milk production, which was also observed in G-2.

Figure 9 shows the percentage of response to serovars from Mo 1 to Mo 9 with greater prevalence of serovars Hardjoprajitino, Pyrogenes and Pomona. Regarding

the 49 animals in group G-1, **Figure 10** shows the average prevalence of serovars, the decrease in productivity in G-2 at moments 4, 5, 6, 7 and 9 and in G-1 at moments 5 and 9. There was a decrease in the pregnancy rate at moments 3, 4, 5, 6, 8 in group G-2 and at moments 5 and 9 in group G-1.

4. Discussion

Where of response to a single serovar, which occurred in 238 samples, serovar Hardjoprajitino was obtained in 67.4%. Pyrogenes in 41 (11.6%), Pomona in 29 (8.51%) and Wolffi in 18 (5.09%). Hardjo is the serovar most commonly found in cattle, the species considered its primary maintenance host [3]. Serologically identical but genetically distinct types of serovars Hardjo exist: *L. interrogans*, serovar Hardjo, type Hardjoprajitno and *L. borgpetersenii*, serovar Hardjo, type Hardjobovis [11]. The chief cattle infectant serovars are Hardjo, Pomona, Gripptyphosa, Icterohaemorrhagiae, Wolffi and Canicola [12]. These were found in the present study at different times with varied percentages both in G-1 and in G-2.

Results show that the serovars are practically the same, seroprevalence varying in both groups with G-2 displaying the greatest differences for most serovars. This shows the importance of environmental contamination and indirect transmission, mainly by water and food. According to Lenharo et al. [13], this serovar is commonly found in wild mammals and these can act as sources of soil contamination and animal infection.

Serovars Bratislava, Djasiman, Hebdomadis, Icterohaemorrhagiae, Pomona and Tarassovi are considered incidental in cattle and indirect transmission is associated with contact with an environment contaminated by leptospires mainly from wild species or other domestic species [8]. On the other hand, serovars Pomona, Grippotyphosa and Icterohaemorrhagiae are frequently identified in incidental infections in cattle and their transmission related to pigs, rodents and wild animals [4, 14]. Bovines can host incidental serovars for an uncertain period [15].

Serovar Hardjoprajitino is responsible for decreases in cattle milk production and conception rates. Also commonly found in pigs, Wolffi is antigenically similar to Hardjo and cause of reproductive disorders and abortions in wild animals and therefore a source of environmental contamination. Serovar Pyrogenes is frequently found in *Rattus norvegicus* and can be considered an incidental contaminant for cattle [6]. Infection by Hardjobovis is frequently observed in cattle in several countries in subclinical forms associated to abortion, while serovar Hardjoprajitno, found in some countries, is characterized as more pathogenic and leading to reductions in milk production and reproductive problems [16].

Serovars Hardjobovis and Hardjoprajitino are adapted to cattle and cause the reproductive and the sudden milk production decrease syndromes. The first is related to serovar Hardjobovis and is characterized by miscarriage, stillbirths, infertility and weak calves. The latter is due to serovar Hardjoprajitino, characterized by udder flaccidity and a sudden decrease in milk production lasting from 2 to 10 days with changes in its consistency and colostrum [17].

Where response was observed for two serovars, the predominance of Hardjoprajitino serovars in 96 of the samples (41.9%), Pyrogenes 53 samples (23.1%), Pomona 20 samples (8.73%), Hardjobovis 18 samples (7.8%), Wolffi 16 samples (6.78%) and Guaricura in 13 samples (5.67%) was noted. The serological response can be influenced by the cross-detection between serovars of the same serogroup. Serovars Pomona, Grippotyphosa and Icterohaemorrhagiae are frequently identified in incidental infections in cattle and their transmission is related to pigs, rodents and wild animals [4, 14].

Predominance of Hardjoprajitino serovars with 25 samples (25.7%), Pyrogenes 21 (21.6%), Pomona 12 (12.37%), Wolffi 15 (15.4%), Copenhageni 9 (9.27%), Guaricura 6 (6.18%) and Hardjobovis 5 (5.15%) samples was noted. Cattle is considered maintenance host for serovars Hardjoprajitno and Hardjobovis which are transmitted by urine associated with reproductive failures [2, 7]. Cross-reactions occur between different serogroups, mainly in the acute phase of the disease [17, 18]. The serovar Icterohaemorrhagiae found in the present study falls within the One Health concept mainly due to the presence of rodents [19]. On the other hand, participation of serovar Pyrogenes among the serovars that stand out at different times is highlighted in G-1 for moments 4, 5 and 6. According to Lenharo et al. [20] this serovar is commonly found in wild mammals, which can contaminate the soil and can infect animals.

Pregnancy decreased at moments 3, 4, 5, 6 and 8 in G-2 and at moments 5 and 9 in group G-1. In a study with 25 dairy herds, totaling 500 cows, 32% of the herds were positive for the *Sejroe* serogroup. Of the 500 cows studied, 48 (9.6%) were sera reactive, 38 (7.6%) with 400 IU titers and 10 (2%) ≥ 800 IU. Estrus repetition was the most reported reproductive problem and strongly associated with leptospirosis [21]. Milk production decreased in G-2 at moments 4, 5, 6, 7 and 9 and in G-1 at moments 5 and 9.

Seroprevalence, milk production and pregnancy rate are influenced by environmental contamination from animal urine, particularly regarding serovar Hardjo. This serovar decreases fertility, while Hardjoprajitino is related to milk production, which is in line with the reduction in liters of milk at moment 6 [16]. Increased rainfall contributes to the spread of the agent in both groups. This is a relevant aspect to be considered in zoo-sanitary management in relation to bovine leptospirosis since the environment has an important role in the chain of transmission of the disease [13]. The triad is thus complete: animal, infectious agent and environment plus human involvement which characterizes the idea of One Health since the disease is common to humans and animals.

With regard to the animals in G-2 and the production of liters of milk, there is a decrease at moment 6, in February, moment 7 in March and moment 9 in May. The lower milk production in these months may be related to the greater environmental contamination by lepspiras and therefore a reduction in output, possibly influenced by serovar Hardjoprajitino.

Pregnancy rates at Mo 5 were 75.5% in G-1 and 76.9% in G-2. Although figures were close, G-2 saw a slight increase. Milk production decreased in both groups. Preganancy rates and milk production are probably related to infection by serovar Hardjoprajitino. Rainfall increased significantly in October, November, December, January and February possibly favoring cross-contamination between the two groups.

The dog is the natural host of serovar Canicola and the brown rat (*Rattus norvegicus*) of serovars Icterohemorrhagiae, Copenhageni and Pyrogenes. Serovar Pomona has pigs, cattle and possums as its natural hosts while Grippotyphosa is found in the kidneys of wild animals such as rats, hares, martens and hamsters [22]. These animals can be sources of infection for cattle [3].

Hardjoprajitino is the serovar prevalent in cattle and responsible for decreased milk production and pregnancy rates, a fact observed in the present study. Pomona and Wolffi are adapted to swine and bovine species but Wolffi is frequently found in pigs and can also cause abortion in the final third of gestation, birth of weak fetuses and decreased conception rates [19].

Serovars Hardjoprajitino and Hardjo are the ones most frequently found in cattle and may cause productive and reproductive disorders [17]. Pomona is most commonly found in swine, which is adapted, however, it can infect cattle.

Pomona is most commonly found in swine, to which it is adapted, it may infect cattle. Serovar Pyrogenes is found in the *Rattus norvegicus* species, implying a potential for environmental contamination. Rodent control and site management measures like waste removal and swamp land drainage are biosafety measures for preventing the spread of this serovar. Despite low at the studied moments, the occurrence of serovar Icterohamorrhagiae should be noted and its adaptation to the rodent species stressed [5, 13].

In order to investigate the effects of rainfall on leptospira infection in cattle, 582 animals were selected and samples from 362 of these collected in the rainy season and from 220 in the dry season. In the rainy season, seropositivity to MAT was 43.6% (158/362) and in the dry season 31.8% (70/220). The Sejroe serogroup predominated (54.8%; n = 125/228), the Javanica serogroup (16.2%; n = 37/228), Icterohaemorrhagiae (7.5%; n = 17/228) and Tarassovi (7.0%; n = 16/228). Seropositivity for incidental serogroups was more frequent in the rainy season (50.0%) than in the dry season (34.3%; $p \leq 0.0001$) [23], reinforcing the environmental aspects of leptospirosis maintenance in cattle herds.

Reproductive failures such as early embryonic loss and consequent estrus repetition are increasingly associated with leptospiral infection. Although these failures are frequently associated with several factors, two studies with cattle revealed a strong association of estrus repetition with seroreactivity for the serogroup *Sejroe* [12, 21]. Contrary to the results obtained, according to Faine et al. [17] Hardjoprajitino is associated with decreases in milk production.

In the present study a greater participation of the serovar Hardjoprajitino, serogroup Sejroe, was also observed however the correlation between milk production and pregnancy rates in both G-1 and G-2 had no statistical significance with $p > 0.05$. A limiting aspect is the impossibility of comparing the results of both the dynamics of antibodies and those of milk production and pregnancy rate, as in the present study, since no similar research with two groups of animals living under the same environmental and management conditions on the same property can be found in the literature.

Although in the present study there was no statistical association ($p > 0.05$) between milk production and seropositivity in both groups, except for the months of May and August, which may be associated with a drop in temperature, when results were analyzed for each groups separately, G-1 showed a decrease in pregnancy rate at moments 5, 6, 7, 8 and 9 and in milk production at moments 5, 6, 7, 8 and 9, related to January (Mo 5) 161.6 mm (Ciagro – Centro Integrado de Informações Agrometeorológicas) and February (Mo 6) 363.3 mm rainfall. Those were months of high rainfall favoring environmental contamination. In G-2 the pregnancy rate decreased at moments 2, 3, 4, 5, 6, 7 and 8, October (Mo 2) 234.4 mm, November (Mo 3) 135.2 mm, December (Mo 4) 137.8 mm, January (Mo 5) 161.6 mm and February (Mo 6) 363.3 mm, all months with high rainfall. Productivity decreased at moments 4, 5, 6, 7 and 9.

There was no statistical association between pregnancy rate and seropositivity, $p > 0.05$ in either group. There was also no statistical association ($p > 0.05$) between milk production and positivity in either group except in May and August, when there was a decrease in milk production which may be related to food management, temperature drop and health of the mammary gland as a result of probable cases of mastitis. The property carrying out somatic cell counting (SCC) of milk samples from the expansion tank but not from individual animals was a limiting factor.

According to Ellis [4], bovine leptospirosis is most often caused by strains adapted from the serogroup Sejroe. bovine leptospirosis is most often caused by

strains adapted from serogroup *Sejroe*. In these cases, disease acute phase may be subclinical except for infections in lactating cows where agalactia may occur. Clinical cases are less frequent and can represent outbreaks, the disease then characterized by abortions at any time during pregnancy [24, 25], albeit more frequent in the average period of pregnancy [3].

Seropositivity for leptospira and clinical cases of leptospirosis are often associated with environmental risk factors, such as rain and floods [26]. For the *Sejroe* serogroup, specifically the Hardjo genotypes, adapted to cattle, direct animal-to-animal transmission is more common than indirect transmission from environmental contamination. On the other hand, infections by incidental serovars by serogroup Icterohaemorrhagiae or Pomona lead to renal excretion. Transmission in incidental infections is more dependent on the presence of other host species and environmental factors, especially accumulated water [4].

Research on leptospiral DNA in the vaginal secretion of apparently asymptomatic cows reinforces the belief that in addition to environmental contamination infection can occur from female to male through vaginal discharges and secretions during natural mating [27]. This can hamper control programs by maintaining infection and disease endemic in the property.

For the Copenhageni, Pomona, Wolffi and Prajitino serovars, frequency of positive titers greater than 800 IU was significant, with $p < 0.05$, in the comparison of positive reagent greater than negative reagent. Cattle infected with adapted strains, including those related to cases of agent isolation [28] the property, often have low antibody titers [10].

Although leptospires can be detected in the urine of cattle infected with adapted strains [10], leptospiruria is intermittent and not very intense [4, 29]. Serovars Pomona, Grippotyphosa and Icterohaemorrhagiae are frequently identified in incidental infections in cattle and their transmission is related to pigs, rodents and wild animals [4, 14].

Infection transmission by incidental serovars is more dependent on the presence of other host species and environmental factors. A high percentage of isolation of the serovar Hardjo from the genital tract of cows is emphasized, suggesting tropism for that region [16]. Also, according to Ellis [4], as previously mentioned, the genotypes of Hardjo serovars are adapted to cattle and associated with the chronic reproductive form of leptospirosis.

The farm where the present study was developed carries out vaccination against leptospirosis every four months and elevated titers such as 800 IU, 1600 IU and 3200 IU were found. In vaccinated cattle, post-vaccination IgM and IgG titers are low (between 100 and 400) and transient between four to six months after vaccination [3]. This fact reinforces the possibility of the higher titers having been produced in response to infection.

With regard to milk production and pregnancy rates, Ellis [16] demonstrated relationship with serovar Hardjoprajiino, a result also found in the present study which corroborates the findings of reductions in milk production and pregnancy rates at the moments when Hardjoprajiino was the most detected serovar. Comparative discussion regarding data from the literature in similar studies is hindered due to the scarcity of research on infection dynamics with different groups of animals. The present study showed that the several serovars are maintained in the two groups of animals (G-1) and (G-2), that seroprevalence is also variable, and that some serovars show greater importance in these groups. It can also be observed that milk production and pregnancy rates decreased at those moments when the frequency of a given serovar, like Hardjobovis, increased.

5. Conclusions

The serovars were practically the same, seroprevalence varying among the animals of the two groups, most of them showing greater variations in G-2, indicating possible environmental contamination and indirect transmission especially through water and food.

Seroprevalence, milk production and pregnancy rates were influenced by the contamination of animals in the environment as well as by the increase in rainfall levels and the possibility of leptospires in the urine of infected animals, considering the two groups G-1 and G-2, and the serovar Hardjoprajitino was the most prevalent, 36% in G-1 and 59.5% in G-2, showing a relationship between positivity and decreases in milk production.

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Ethics committee approval

This study was approved by the Animal Use Ethics Committee (CEUA) of FMVZ-UNESP/Botucatu, SP, process nr. 0154/2019, September 11, 2019.

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Chapter 6

Antimicrobial Resistance in Staphylococci Special Emphasis on Methicillin Resistance among Companion Livestock and Its Impact on Human Health in Rural India

Sweta Jangra, Sandhya Khunger and Debasish Chattopadhya

Abstract

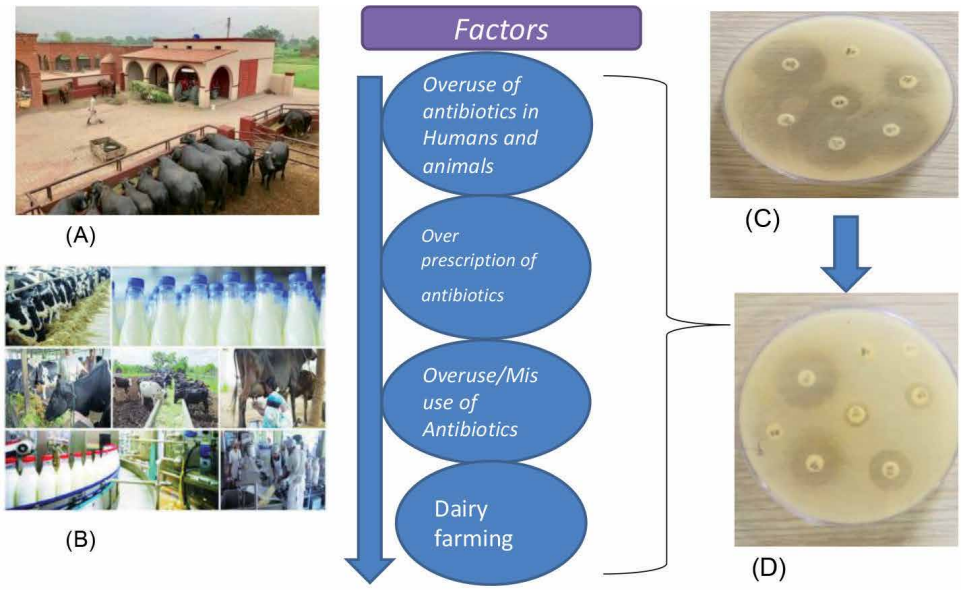
Antimicrobial resistance (AMR) is a global threat worldwide. Inappropriate and irrational use of antibiotics are the responsible causes for the development of AMR in the pathogenic microorganisms. In the developing countries like India the data encountered a higher burden of resistance in the rural communities. In such scenario the AMR may lead to difficulty in treatment of various ailments among human as well as companion livestock. In India cows and buffalo are considered as companion livestock. However the definition of companion livestock is slightly different in the developed countries. Most of the rural population in India is dependent on the livestock for their livelihood as the dairy farming in the rural community may contribute in the financial status of the rural population. *Staphylococcus aureus* (*S. aureus*) is one of the foremost causative agent of skin and soft tissues infections among humans as well as in companion livestock. The situation is further complicated by methicillin resistance in *S. aureus*. The carriage of MRSA by humans and companion livestock may lead to further AMR spread to the community. In the civic health point of view, it is important to initiate appropriate interventions to tackle the problem at the rural population.

Keywords: Companion livestock, Antimicrobial resistance, *Staphylococcus aureus*, Methicillin resistant *S. aureus*, Rural India, LA-MRSA

1. Introduction

Antimicrobial resistance (AMR) is a global threat and is of a major public health concern globally [1]. AMR is defined as a state when microbes including bacteria, virus, fungi and parasites no longer respond to the drugs thereby increasing their risk of disease spread, causing severe illness and death (**Figure 1**) [2].

AMR can spread through 'horizontally' by different transformation method in which bacteria transfer a part of their genetic material with another bacteria, or the



A) Companion livestock residing in same premises with human. ***Source: https://www.pinterest.com/pin/739786676278783154/***
B) Dairy Farming in India. **Source: https://www.startyourindustry.com/tag/dairy-farming-in-india/**
C) Antibiotic sensitive strains
D) Antibiotic resistant strains

Figure 1.
Graphical abstract showing AMR spread in rural India.

spread may be 'vertically' where AMR genes are continuously transferred from one generation to the next and so on [3].

Although inappropriate and irrational use of antimicrobial agents in animals has been considered to be responsible for the emergence of drug-resistant pathogens [4].

Making the treatment of infectious disease with antimicrobial agents challenging contributing to the community with respect to infectious disease burden. World Health Organization (WHO) declared AMR as one of the top 10 public threats as a result this may lead to high disease load in India which is considered as maximum in the world [2]. The current situation is reflected by high rate of AMR against several infectious diseases including urinary tract infections (UTIs), soft tissue infections, diarrhea and sepsis. In addition the rapid spread of penicillin-resistant bacteria (Methicillin resistant *Staphylococcus aureus* (MRSA) superbugs are also alarming.

Many sustainable development goals framework in the rural India have included "AMR indicator" that monitors the frequency of bloodstream infections caused due to antimicrobial resistant organisms including *S. aureus (S. aureus). S. aureus* is a part of human skin flora and a common infection causing agent implicated in acute food poisoning episodes, scalded skin syndrome,impetigo, cellulitis, folliculitis toxic shock syndrome, and furuncles [5]. Reports in the literature suggest that patients with MRSA infections are 64% more likely to die due to unresponsiveness of beta-lactam antibiotics and vancomycin which are the drug of choice for treating various ailments produced by Staphylococcus [6].

Notably *S. aureus* infections have raised concerns these days due to increasing antimicrobial resistance (MRSA). In rural India the antibiotic usage in humans and in the livestock is high with no regulation on the usage of antibiotics which may be a leading cause of antimicrobial resistance [7]. Indian Network for surveillance of Antimicrobial Resistance (INSAR) along with WHO monitors AMR and its magnitude in India. Their major goal is to focus on AMR patterns of various antimicrobial resistant microorganisms including *S. aureus*.

Rural community of India is dependent on companion livestock for their livelihood as livestock sector is major part in developing their socio-economic status. Livestock sector found to be responsible for about 6% to the gross domestic product (GDP) and 25% to the agricultural GDP of India [7, 8].

The dairy farming is a common practice which have a positive impact on the rural system [9]. Thus, the rural population is much exposed to acquisition of AMR from livestock. Most reports on AMR in human associated with companion livestock have been confined to enteric organisms. However, staphylococcal infections in livestock is palpably common in India which include staphylococcal infections viz. Bovine mastitis, wound infections and udder impetigo ultimately affecting the public health and economy [10].

The present report attempts to review the problem of AMR in Staphylococci in livestock animals and their possible transmission to human.

2. Antimicrobial resistance

AMR occurs when pathogens change genetically over time so as to become resistant to antimicrobials. As a result, the antimicrobials become ineffective against microorganisms and the infections remain persistent in the body. Antimicrobials including antibiotics, antivirals, antifungals and antiparasitic are the form of drugs that used to prevent and treat respective infections in humans and animals. Microorganisms that develop AMR are sometimes referred to as "superbugs" [2].

AMR has developed as one of the foremost community well-being issue of the 21st century that threaten the health care system due to ineffective prevention and treatment measures against wide range of infections. The problem of AMR in bacteria is of great concern. Over several decades, bacteria are involved in common or severe infections along with resistance towards variety of antimicrobials used against variety of ailments caused by bacteria. It is quite difficult to underestimate the impact of AMR in terms of death rate and community health. Thus, a much-needed action plan is required to estimate such developing global issue in health care settings as well as in the community.

In natural environment, antibiotic resistance occurs in antibiotic-exposed microorganisms over time. Susceptible bacteria are killed or inhibited under the bactericidal effect of antibiotics. However, microorganisms that have acquired antibiotic-resistant genes due to overuse, misuse and unsuitable (inadequate dosing inappropriate choices and weak acceptance of updated treatment guidelines) of antibiotics have a greater chance to survive and proliferate. This is how the antimicrobial resistance is facilitated. In a developing country like India, the excessive prescription by general practitioners due to similarity of clinical presentations in viral or bacterial etiology can be seen. In addition the diagnostic uncertainty, self-medication and easy availability of antimicrobial drugs without a proper prescription is also have significant root cause in the inappropriate use of antibiotics [11].

2.1 Methicillin resistance *S. aureus* in human

S. aureus has been reported as a significant pathogen of human subjects. Sir Alexander Ogston in 1880 firstly reported *S. aureus* as a major cause of wound suppuration. Skinner and Keefer provided the first evidence of *S. aureus* virulence in 1941 and reported 82% mortality rate due to *S. aureus* associated bacteremia. In 1960s, the introduction of β-lactam antibiotic revolutionized the clinical care system and decreased the mortality rate associated with *S. aureus* bacteremia markedly to 27% with its widespread usage [12, 13].

S. aureus is one of the most studied troublesome resident and notorious pathogen in the human skin. *S. aureus* is a member of genus Staphylococcus that consist of fifteen different strains with differential molecular data. The status of *S. aureus* as a commensal is highly controversial. Several reports suggest that at around 30% of the nasal cavity flora of humans carries *S. aureus* or coagulase positive *S. aureus* (CoPS) species [14, 15]. Asymptomatic healthy humans and animals could harbor multiple species and strains of staphylococci [16].

Methicillin was the first semisynthetic penicillinase-resistant penicillin introduced in 1961 for treatment of Staphylococcal infections. Soon after its introduction in clinical practice, reports of methicillin-resistant isolates encountered [17].

The dissemination of MRSA have a great impact on both rural society as well as on hospital settings [18]. with all evidences centers for diseases control (CDC) declared methicillin resistant as a consequential issue to public wellness [19]. Thus, knowledge regarding the mechanisms of methicillin resistance in *S.aureus* has great clinical and epidemiological importance.

Based on the antimicrobial susceptibility, MRSA exists in three forms i.e., community associated MRSA (CA MRSA); health care-associated MRSA (HA-MRSA) and livestock associated MRSA (LA-MRSA). The difference among all the three forms lies with their clinical features, molecular biology, antibiotic susceptibility pattern and treatment measures. CA-MRSA and HA-MRSA are the major form of infections. MRSA infections are endemic in India with an incidence rate of 25% in Western regions and 50% in South regions of India. However in India, CA-MRSA and LA-MRSA are more prevalent as compared to HA-MRSA. The major concern arises when the isolation rate of CA MRSA enhanced as these species lead to replace HA-MRSA among hospitality settings which in turn build infection preventive and control measures slighter effective and significant for reducing the isolation rates of MRSA in healthcare settings [20].

2.2 LA- MRSA

The problem of methicillin resistant *S. aureus* associated with livestock is long recognized as LA-MRSA. A-MRSA is entirely different from the other two forms (Hospital acquired, and community acquired). However LA-MRSA is not associated with health-related concerns in animals, but it also affects human health if they are in contact with them. In India most of the population is dependent on livestock for their livelihood. Transmission of the methicillin resistant strains from animals to humans or vice versa and may lead to any infection.

The MRSA carriers (animals/humans) are considered as colonized. Harbored animals may pretend as MRSA pools. Persons who lives and comes in direct interaction with MRSA colonized livestock are at high risk of being harbored/colonized by LA-MRSA. This could be a leading cause of transmission of LA-MRSA to further subjects subsequently transmitting the disease in the community at very high extent [21].

LA-MRSA is grouped under zoonoses which is described as the naturally spread infections among “animals and humans” as per the definition by WHO expert committee, 1951. Based on the prevalent direction of transmission between humans and other vertebrates, ‘zoonosis’ is also considered as ‘Anthropozoonosis’ which is suggestive for the transmission of humans to animals. Colonization with LA-MRSA may result into various ailments among human as well as in animals.

MRSA is commonly associated with bovine mastitis and the first evidence of mastitis was recorded in dairy cattle in 1972. Since then, MRSA colonization has been reported in the domestic animals namely cows, dogs, horses, sheep, cats and pigs [22, 23]. Mastitis with *S. aureus* features the genotypes of hugely deviating

mecA gene, termed as *mecC* in a type XI SCCmec or ST398 MRSA with SCC*mec* types IV andV. The appearance of LS MRSA (MRSA*mecC* and CC398) carrying SCC*mec* types (IV and V) and *mecC* were isolated firstly from pigs afterwards in cattle, pet animals, lineal calves, chickens, horses, fauna and human subjects in adjacent vicinity in those tame livestock's [24]. The clone type ST-398 of LA-MRSA, is commonly detected in European and North American countries whereas ST-9 primarily in the Asian region. The SCCmec type IV and V have to be found as co-resistant towards tetracycline and lincosamide, which are moderated by *tet* and *erm* genes successively [25]. A special attention is needed to be given for the patients with SARS-CoV requiring ventilation support as this pandemic may result into markedly rise in MRSA infection [26].

Due to highest milk production yield in India in the world and involved in dairy production results in high prevalence of LA-MRSA. The zoonotic potential of many bacterial strains among humans and animals are increasingly being isolated in Europe with much of the industrialized world [27, 28]. Most of the cattle's in India shared same residential premises with humans hence are more prone to transfer MRSA to the humans who are in close contact with them. The detection of MRSA special emphasis to cattle with sub/clinical mastitis is highly concerned from public health sentiments as the cattle looks all right even if they are colonized with reservoirs with MRSA [27, 28]. However the situation is something different in India as livestock's are considered as a family members due to their same residential premises sharing.

In addition to the above Jully G Tiwari from Assam showed that all the family members of the dairy farm worker were suffering from an identical type of cutaneous disease as of the bovine infection. The antibiotic susceptibility pattern (AST) of all biotype A strains from animals' origin were found alike to that of the biotype A strains isolated from the humans associated with the day care activities with the animals. In addition to the previous findings the rate of resistance towards commonly used antimicrobial agents in different ailments were also found markedly higher among the biotype C strains originated from human subjects than the biotype C strains from the animal origin. In addition many strains from animals and human origins revealed similar antimicrobial susceptibility testing patterns against various tested antimicrobial agents. [29]

The prevalence of Staphylococcus species was found in the mastitis cases of dairy animals as 45% (95% CI, 39–50%) based on the previous meta-analysis in India reported by Krishnamoorthy et al. [30].

2.3 Rural/urban areas and associated risk of MRSA in India

As per Census of India 2011, the definition and criteria for characterization of urban and rural area was as follows;

i. Minimum population of 5,000;

ii. A minimum of 75 percentile male population should be enrolled in non-agricultural tasks;

iii. Population weightage of at least 400 people per sq. km.

If an area justify all the above requirements, that will be classified as "Urban" however an area is considered as 'Rural' if it is not qualified the criteria of 'Urban' as per the above definition. In the rural communities of India, majority of human subjects are involved in agricultural pursuits and hence agriculture is their major

profession. Thus, this population is at high risk of developing/acquiring MRSA. The acquisition of drug resistant bacteria like MRSA due to contact with colonized animals is a common method for the spread of LA-MRSA. This may be a significant way for acquisition of LA-MRSA by the human population in India.

In developed countries i.e., US, the defining criteria of companion livestock's is different according to that dogs and cats are majorly treated as companion animals whereas in developing countries including India, the livestock's definition is slightly different as buffaloes and cattle's are considered as companion animals in rural population on the basis of sharing of similar residential premises. Livestock's have a significant potential to improvement towards food & nutritional status, agricultural enhancement, reduction in rural poverty and alleviating farm households in India [31–33].

Livestock's fostering is found to be an important concept of the rural financial status, significantly at the family circle extent. However not much data is available on the impartation of animal fostering sector towards the family incomes. In India, livestock raising are mainly emerged as a part of mixed agricultural organizations as they contribute 25–30% of farming GDP outputs. It is estimated that just about 3/4th of the manpower required in livestock's production is significantly donated by women [34]. However the significance of women in livestock building is hugely applauded but the issues related to their authority over the financial inputs from livestock's activities and its probable effects on children's wellness, nutritional status and schooling have not gained further attraction among the empirical writings [35]. According to two assessment surveys that were conducted in 2003 and 2013, by the National Sample Survey Organization (NSSO) that was situational based they reported that livestock cover only farmer households [36].

3. Antibiotic resistance among companion livestock

MRSA was identified for very first time in 1972, from mastitis-affected cows and then MRSA from human origin was isolated from dairy cows [37]. MRSA has been found in almost all tame animals since its first observation. Major associative factor for MRSA found to be wound infections and post-operative skin infections, especially in dogs, cats, horses, and rabbits [38, 39]. In one of the study of milk-producing bovines in Mathura reported that MRSA in India was shown to be *S. aureus* was found in 33.75% of subclinical mastitis and clinical mastitis cases among cattle than buffaloes [40]. Tiwari *et al.* (2015) published *S. aureus* among different specimens from pigs, buffalo, dogs, goats, sheep, camels, and horses with different skin disorders of all companion livestock's [41]. *S. aureus* isolates from healthy cow milk samples were found to be resistant to erythromycin (75%), penicillin (100%), and amoxicillin (100%), but susceptible to cloxacillin (100%), neomycin (100%) oxacillin (100%), and ciprofloxacin (83.33%) [42]. MRSA study by Kumar *et al.* (2017) was shown to be penicillin and oxytetracycline resistant (88% each), cefoxitin resistant (75%), cotrimoxazole resistant (62%), and amoxyclav resistant (62%) in antimicrobial susceptibility screening conducted in study on 136 skin and nasal samples collected from cows and buffaloes. Vancomycin resistance was discovered in 3 (16.7%) MRSA isolates from buffalo isolates [43].

3.1 Transmission of antimicrobial resistance from livestock to humans

Livestock's colonization with antimicrobial resistance organisms and human infections due to drug resistant organisms are appearing as a potential threat worldwide. MRSA is now regarded as an emerging zoonotic agent. Over the last

two decades, MRSA has been noticed in the population. Novel MRSA strain known as livestock-associated MRSA (LA-MRSA) has been discovered in farmers and fodder producing animals. [44, 45]. In previously literature, MRSA sequence form was known as non-typable MRSA (NT-MRSA) lacking property of typed using pulsed field gel electrophoresis(PFGE) with SmaI. ST398 isolated as a prevalent sequence type in the agricultural animals, especially pigs and veal calves, as well as persons who worked with these animals [46]. Carriers of MRSA are at risk of developing an MRSA (wound) infection following skin damage (scarification) or surgery. The mode of MRSA transmission from animal to human is frequently inferred from parallel genetic observations including isolates obtained from *in-vitro* experiments [47, 48]. Another study revealed that incorporation of mecA gene encoding penicillin-binding protein 2a into the *Staphylococcal* cassette chromosome *mec* (SCCmec), are considered as factors in the drug resistance of *S. aureus* strains. There is evidence that methicillin-sensitive *S. aureus* strains inherited the SCC mec factor from coagulase-negative staphylococcal strains and subsequently became methicillin-resistant [49].

Reports on the carriage rate of pigs and the farmers associated with those pigs in Netherland divulge a high isolation of MRSA ST398 whereas the same sequence lineage has been observed in the dirt samples from the pig propagation space and eatable samples in Austria. In China, LA MRSA ailment is considered as an professional pitfall for farm workers, LAMRSA in the animal handlers may ranges from region to region i.e., 19.2% in Taiwan, 5.5% from Malaysia and 15% in China [50, 51]. Globally, MRSA percentage increased by 20% in all WHO sectors while 80% increase in few countries [52].

Factors - The emergence and spread of LA-MRSA is attributed to antimicrobial resistance upsurge. However hygiene along with under-described or poorly investigated factors i.e., farm dimensions, cultivation system, use of bioinoculants, and in-feed microelements also contributing the emergence of AMR [53]. Other than *S. aureus* some species of Coagulase negative staphylococci (CoNS) may also be transferred from animals to humans in contact with them, evidence found from a study (Jangra et al. 2018) that revealed some pathogenic strains of Coagulase negative staphylococci (*Staphylococcus warneri* and *Staphylococcus scuiri*) that are announced as a particular pathogen linked with animal origin however their isolation from the human subjects associated with them were also be detected. These results further strengthening the probability for the transmission of such pathogenic species from the domesticated animals [54].

4. Conclusion

We can conclude from the above literature that methicillin resistance MRSA freight among humans and animals seems a great concern towards effectiveness of antibiotic treatment. AMR may spread due to indiscriminate use of antibiotics without prescription, supervision and guidance of general practitioner in the developing countries including India. However in the rural India antimicrobials use in dairy animals for growth promotion and food producing animals for the cure of any ailments are probably major contribution towards the altogether issues of the resistance. Policies governing prescriptions of antibiotics should be strictly followed in animal farming to avoid AMR and associated infections. Clinicians and healthcare institutions should be encouraged to espouse appropriate guidelines about the use of antimicrobials regarding MRSA affected patient treatments in command to containment of community acquired staphylococcal infections. Farmers should be made aware about the dosage, length and

administration of the treatment and withdrawal period of antibiotics prescribed by the clinicians in order to cure any infection.

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Conflict of interest

"The authors declare no conflict of interest.

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Chapter 7

CD4+ T Cell Responses to Pathogens in Cattle

Anmol Kandel, Magdalena Masello and Zhengguo Xiao

Abstract

Helper CD4+ T cells are essential in shaping effective antibody response and cytotoxic T cell response against pathogen invasion. There are two subtypes of pathogen-specific helper T cells in mice and humans; type 1 (Th1) and type 2 (Th2), with Th1 producing interferon-gamma (IFNγ) and Th2 producing interleukin-4 (IL-4). While effective Th1 controls intracellular pathogens like viruses, efficient Th2 controls extracellular pathogens like most parasites. However, the most predominant CD4+ T cell subtype in cattle is Th0, which produces both IFNγ and IL-4, and only exists in small amounts in mice and humans. Moreover, in many bovine infections, both IFNγ and IL-4 were detected in the blood and both antigen-specific IgG2 (Th1 associated bovine antibody) and antigen-specific IgG1 (Th2 associated bovine antibody) were upregulated in the serum, suggesting bovine CD4+ T cell responses may vary from those in mice and humans. How bovine CD4+ T cell differentiation differs from that in mice and humans and how some critical bovine pathogens regulate immunity to establish chronic infections are largely unknown. This chapter summarizes current literature and identifies the knowledge gaps to provide insights into future research in the field.

Keywords: bovine, CD4+ T cell differentiation, antigen-specific clones, Th0 responses, pathogens, chronic infections

1. Introduction

CD4+ T cells, also called helper T cells, are important regulators of adaptive immune responses, which are antigen-specific and critical in protecting animals from pathogen infections. The control of intracellular pathogens, such as viruses, primarily depends on antigen-specific CD8+ T cell response, whereas antibodies (produced by B cells) or humoral immune responses are mostly responsible for the control of extracellular pathogens such as most bacteria and parasites. CD4+ T cells are the lynchpin in shaping both CD8+ T cell and antibody responses [1, 2].

Common lymphoid progenitor cells migrate from the bone marrow into the thymus for further development and maturation into T cells. Inside the thymus, these progenitor cells proliferate into a large pool of T cells, with each expressing a unique T cell receptor (TCR) through a genetic recombination. After TCR recombination, T cells must go through two selection processes, and only a fraction of them pass through these selections and become either CD4+ or CD8+ T cells [3]. Surviving CD4+ T cells then exit the thymus as naïve CD4+ T cells but without the ability to help CD8+ T cells and B cells. To become fully functional, naïve CD4+ T cells need to become activated and differentiated into specialized effector subtypes; helper

type 1 (Th1) to facilitate CD8+ T cell responses, and helper type 2 (Th2) to facilitate antibody responses [4]. Naïve CD4+ T cells constantly survey secondary lymphoid tissues to detect pathogens through their antigen-specific TCRs [5]. As opposed to antibodies, which bind directly to pathogens or their derivatives, TCRs can only recognize short chains of amino acids (derived from pathogens) that are presented by major histocompatibility-II (MHC-II) expressed on antigen presenting cells (APCs) [2]. This recognition process provides the 1st signal required to activate naïve CD4+ T cells. Along with the 1st signal, APCs also offer co-stimulation as the 2nd signal and cytokine signaling, as the 3rd signal, to the naïve CD4+ T cell. Combined, these three signals coordinate CD4+ T cell differentiation into distinct effector subtypes with different helper functions [2].

Studies in humans and mice have identified numerous helper subtypes, including: Th1, Th2, Th3, Th9, Th17, Treg, and Tr1 [2, 6]. Among these, Th1 and Th2 are considered to play major roles in defending the host from pathogen invasion [7–9]. Th1 cells help CD8+ T cells to gain killing functions, which leads to apoptosis of infected cells and induces Interferon gamma (IFNγ) mediated immunity [10–13]. On the other hand, Th2 cells help B cells differentiate into plasma cells, which produce pathogen-specific antibodies [14]. Antibodies or humoral immunity contribute to the control of extracellular pathogens by mechanisms like neutralizing toxins, preventing bacterial attachment to the host cell, and stimulating basophil and mast cells to release toxic chemicals that induce the expulsion of large gastrointestinal parasites [15, 16]. Although antibodies are mostly responsible for controlling extracellular pathogens, they can also play important roles in cell-mediated killing of intracellular pathogens [17]. For instance, during intracellular infections in mice, Th1 cells help B cells become plasma cells that secrete antigen- specific immunoglobulin subtype G2a (IgG2a), which in turn can help killing infected cells through antibody dependent cytotoxicity (ADCC) [18, 19]. In short, Th1 is responsible for control of intracellular pathogens mostly through shaping CD8+ T cell responses and Th2 is for control of extracellular pathogens through antibody responses. In addition, antibodies can be involved in both Th1 and Th2 responses, but with unique subtypes, such as IgG2 for Th1, and IgG1 for Th2 in cattle. This will be discussed further in Section 2.

There are many similarities in the immune system across species. Therefore, knowledge generated from the research in mice and humans has been extensively applicable to study immune responses in cattle [20–23]. In the past several decades, however, unique features have been discovered in the bovine immune system that are not shared with that of mice and humans, such as high prevalence of circulating γδ T cells [24], production of IL-10 by γδ T cells [25], regulation of CD4+ T cell activation by neutrophils [26], which are able to secrete IL-10, and high prevalence of hybrid helper T cells (*i.e.,* co-express both Th1 and Th2 cytokines), which is relatively low in humans and mice [22, 27, 28].

Cattle industry suffers billions of dollar's losses annually due to infections, and many of the commercially available vaccines for cattle are not fully effective [29–32]. Understanding the mechanisms underlying bovine CD4+ T cell differentiation, which seems to be partially different from that of mice and humans, is critical to identify novel strategies to achieve more effective immunity after vaccinations, such as through generating strong Th1 responses against intracellular pathogens and Th2 responses against extracellular pathogens. In this chapter, we will summarize the current knowledge and key findings on bovine CD4+ T cell responses, highlight the existing knowledge gaps, and provide some insights on future directions.

2. CD4+ T cells regulate adaptive immunity

Naive CD4+ T cells exit the thymus and search for pathogen-derived antigens presented by APCs in secondary lymphoid tissues (*e.g.,* lymph nodes and the spleen). During infections, pathogens break through barriers (Physical, chemical etc) of the host to establish infection in the local tissues [33]. As a result, the immune system in the host initiates an inflammatory response through recruitment of immune cells such as neutrophils to the site of infection, which secretes inflammatory cytokines and chemokines [34, 35]. These chemokines provide signals for further recruitment of APCs to the site of infection. APCs constantly search for invading pathogens through recognizing pathogen associated molecular patterns (PAMPS) on pathogens by their pattern recognition receptors (PRRs) [36]. For example, Toll-like receptor-4 (TLR-4) on APCs can recognize the lipopolysaccharide (LPS) present on the cell membranes of gram-negative bacteria [37]. After recognition, APCs engulf the pathogen, break it down into small peptides, and finally present the peptides to CD4+ T cells in the secondary lymphoid tissue. Recognition of this peptide–MHC-II complex by the TCRs on the naïve CD4+ T cells provides the 1st activation signal, as shown in **Figure 1** [41]. At the same time, co-stimulatory molecules on the CD4+ T cell surface (*e.g.,* CD28) recognize their corresponding ligands on the APC surface (*e.g.,* CD80 or CD86), which provides the 2nd activation signal [42]. The final and 3rd signal, which occurs simultaneously with antigen stimulation and co-stimulation, is provided by cytokines such as Interleukin-12 (IL-12) or Interleukin-4 (IL-4) that not only enhance the activation process, but also drive CD4+ T cell differentiation into a specific subtype (*e.g.,* Th1 or Th2) [2, 43]. Therefore, APCs can provide all 3 signals to naïve CD4+ T cells, which facilitates their activation and differentiation (**Figure 1**). Pathogens can regulate host helper T cell response through targeting any of the three signals directly or indirectly, which will be discussed in Section 5. Recently, we have reported that bovine CD4+ T cells respond to three signals in a way similar to that in humans and mice [44]. Furthermore, IL-12 and neutrophils can work on bovine CD4+ T cells synergistically to enhance their production of IFNγ [44].

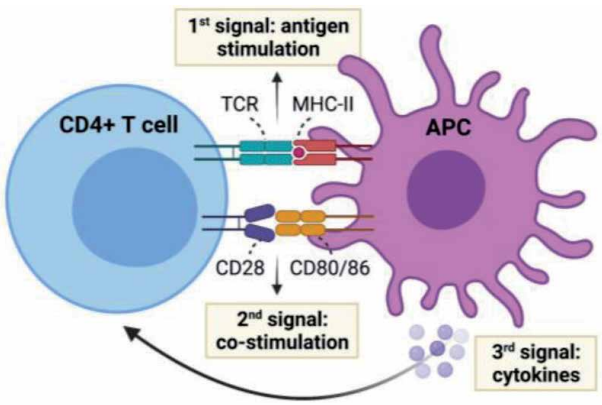

Figure 1.
Three-signal model for CD4 + T cell activation: The 1st signal is provided when TCRs recognize the peptide–MHC-II complex presented by APC; the 2nd signal is initiated when CD28 on CD4+ T cells interacts with CD80/86 on APCs, and the 3rd signal is triggered by cytokines released from the APCs and other cells. CD28/CD80/CD86 interaction is used as an example. This figure was adapted from previous reviews [38–40].

2.1 Th1 cells coordinate CD8+ T cell response to intracellular pathogens

During the infection, the host responds to the intracellular pathogens by inducing cytokines such as IFNγ and IL-12 from APCs like macrophages and dendritic cells (DCs), which further leads to the polarization of CD4+ T cells into a Th1 subtype. IFNγ and IL-12 enhance the expression of transcription factor T-bet, which directs Th1 differentiation in the activated naïve CD4+ T cells (**Figure 2a**) [51, 52]. More specifically, when bound to their receptors on naïve CD4+ T cells, these cytokines induce the activation of transcription factor STAT-1 or STAT-4 respectively, which in turn causes T-bet upregulation [53]. Subsequently, T-bet induces histone modification and binds to the promoter region of Th1-specific cytokine genes, which leads to enhanced expression of IFNγ [51, 52]. In addition, T-bet also inhibits Th2 differentiation by repressing the transcription of Th2 specific genes, such as *GATA-3,* which is the transcription factor responsible for IL-4 expression [51, 54]. Thus, IFNγ and IL-12 induce Th1 differentiation, which leads to IFNγ production and suppression of Th2 differentiation.

One key functions of differentiated Th1 cells is to facilitate the activation of CD8+ T cells by "conditioning" dendritic cells; a process that induces dendritic cell (DC) maturation by modifying their cytoskeletal structure, upregulating co-stimulatory molecules, and by enhancing their migration to secondary lymphoid tissues [55–57]. Once conditioned, these DCs can induce CD8+ T cell activation as shown in **Figure 2(b)**. Although these two processes, conditioning of DCs and activation of CD8+ T cells, might occur simultaneously, some researchers argue that this process may occur in two sequential steps: conditioning DC first, followed by CD8+ T cell activation [56, 58, 59]. Activated CD8+ T cell secretes cytotoxicity-related proteins such as perforin and granzyme-B. While perforin forms pores at the cell membrane, granzyme enters through these pores and cause apoptosis of the infected cell [60]. Additionally, antigen-specific CD8+ T cells can kill infected cells through caspase mediated pathway, when Fas molecules expressed on the infected cells interact with Fas Ligand expressed on the antigen-specific CD8+ T cells [61].

IFNγ is a critical cytokine performing multiple functions to assist Th1 response against intracellular pathogens in mice, humans and cattle [62]. Although many types of immune cells can produce IFNγ including NK cells, DCs, macrophages and B cells, it is the signature cytokine of Th1 subtype [27]. Th1 produced IFNγ plays a critical role in regulating the Th1 response. IFNγ can recruit immune cells to the site of infection and promote anti-microbial activities of neutrophils and macrophages by inducing oxidative burst and production of reactive oxygen species (ROS) [62–65].

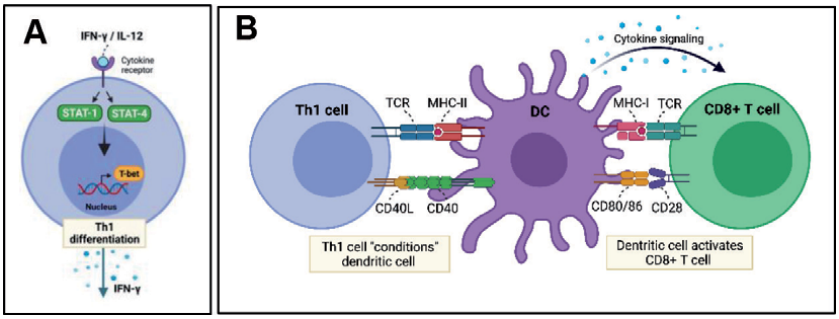

Figure 2.
Th1 help to the activation of CD8+ T cell. A) IFNγ and IL-12 bind to their corresponding receptors on naïve CD4+ T cells during activation, which leads to T-bet expression and Th1 differentiation. This figure was adapted from previous reviews [45–47]. B) Once differentiated, Th1 effector cell conditions dendritic cell, which in turn activates CD8+ T cell. This figure was adapted from previous reviews [48–50].

IFNγ is directly involved in blocking viral replication, as well as enhancing the cytotoxic activity of CD8+ T cells [66, 67]. Moreover, IFNγ can enhance the number, mobility, and cytotoxicity of CD8+ T cells [67, 68].

During infection caused by intracellular pathogens, Th1 produced IFNγ can induce IgG subtype switching in activated B cells. However, this subtype switching may differ among the species. For example, it induces production of IgG2a in mice and IgG2 in cattle but IgG1 and IgG3 in humans (**Table 1**) [18, 69, 73]. These IgG subtypes induced by IFNγ can facilitate multiple mechanisms such as ADCC to kill intracellular pathogens, such as *Coxiella burnetii*, *Listeria monocytogenes,* and *Toxoplasma gondii* in mice [19, 82].

2.2 Differentiated Th2 cells coordinate humoral response against extracellular pathogens

During infections caused by extracellular pathogens, innate immune cells such as basophils, eosinophils, and innate lymphoid cells (ILCs) produce and secrete IL-4 [83, 84]. Together with 1st and 2nd signals, IL-4 signaling on naïve CD4+ T cell upregulates GATA-3 (GATA binding protein-3), a critical transcription factor for Th2 differentiation [85, 86]. GATA-3 knockout mice mounted impaired Th2 responses [87, 88]. When IL-4 binds to its corresponding receptor on the surface of naive CD4+ T cells, it activates STAT-6, which turns on pathways leading to GATA-3 expression (**Figure 3a**) [93, 94]. Consecutively, GATA-3 promotes Th2 differentiation by inducing histone acetylation and enhancing transcription of the IL4 gene [83, 95]. In addition, GATA-3 is capable of suppressing Th1 differentiation by downregulating transcription and expression of molecules such as the IL-12 receptor *β2,* IFNγ, STAT-4, and possibly T-bet [96].

Once differentiated, Th2 cells are capable of activating B cells to produce antibodies that defend the host against extracellular pathogens [97, 98]. During B cell activation, Th2 cells recognize peptide–MHC-II complexes expressed on B cells [99, 100] and provide co-stimulation via CD40L, which are both necessary for B cell activation [101] (**Figure 3b**). Importantly, IL-4 signaling induces isotype and subtype switching of B cells towards IgE and IgG1 production, which are key antibodies for controlling extracellular pathogens in mice and cattle [102].

Although antibodies can assist CD8+ T cell responses during intracellular infections, they play a major role in controlling infections caused by extracellular pathogens [13, 103, 104]. Antibodies can prevent the attachment of extracellular

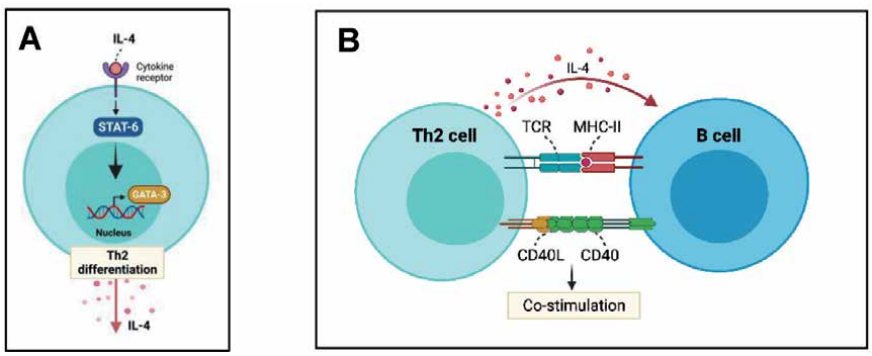

Figure 3.
Th2 help to the activation of B cell. *A) IL-4 binds to its receptor on naïve CD4+ T cells during activation, which induces GATA-3 activation and Th2 differentiation. This figure was adapted from previous reviews [46, 89, 90]. B) Once differentiated, Th2 cells secrete IL-4 and provide antigen stimulation and co-stimulation to a B cell. This figure was adapted from previous reviews [91, 92].*

bacteria to the host cell, facilitate phagocytic killing, and neutralize toxins [13, 105–108]. In addition, different antibody isotypes and subtypes can have different functions. For instance, IgE can bind to both low and high-affinity receptors (FcεRI and FcεRII) on mast cells and basophils, which results in the degranulation and release of chemicals (*e.g.,* histamine, leukotrienes) that either kill parasites directly, or induce hyper-contraction of intestinal smooth muscle to promote their expulsion [109–112].

In addition to IL-4, other cytokines such as IL-5, IL-9 and IL-13 are also involved in the control of extracellular pathogens. For example, IL-9 promotes production of IgE and proliferation as well as maturation of mast cells, which rapidly infiltrate the site of infection [113, 114]. Similarly, IL-5 induces differentiation, maturation, and infiltration of eosinophils to the site of infection [114]. Infiltrated mast cells and eosinophils, when cross-linked by antigen-specific IgE, degranulate (i.e., release histamine and leukotrienes) to kill or expel gastrointestinal parasites. IL-13 on the other hand, plays a significant role in the expulsion of parasites by inducing regeneration of the intestinal epithelium and contraction of smooth muscle cells in the intestine [98, 115]. Nevertheless, there are multiple cytokines involved in the differentiation of Th2 responses, but IL-4 is considered the most critical one.

2.3 Th1/Th2 cytokines induce immunoglobulin class switching during infection

Antibodies produced by activated B cells during infection are classified into five different classes (*i.e.,* IgM, IgG, IgA, IgD and IgE) based on their structure [116]. Among them, IgG is the most abundant in serum, and it has four different subtypes, namely: IgG1, IgG2, IgG3 and IgG4 [116]. Each antibody has two structural segments (heavy and light chains) and two functional segments (F_{ab} and F_c portions). While association of heavy chain with the light chain at the F_{ab} portion forms antigen-binding sites, only the constant portion of the heavy chain constitutes the F_c segment that regulates the effector function of the antibody. During infection, activated B cells undergo isotype or subtype switching, a process that involves switching of F_c segment but not of the F_{ab} segment. Briefly, DNA in B cells contains multiple heavy chain constant genes (or C_H genes) that encode various types of Fc segments [117]. During infections, Th1 and Th2 cytokines provide signals to the activated B cells to select a specific C_H gene for the heavy chain, thus producing a specific isotype or subtype of immunoglobulins with the same antigen specificity [118]. For example, IFNγ can induce subtype switching to IgG2a to enhance the killing of infected cells in mice; similarly, IL-4 can induce switching to IgG1 to promote humoral immunity (**Table 1**) [70–72, 119]. Historically, characterizing serum IgG subtypes was a common practice to define the immune response in clinically ill cattle; the greater concentration of serum IgG2 typically indicated a Th1 response, whereas greater IgG1 indicated a Th2 response. Interestingly, the Th1 induced IgG subtypes may vary among the mice, humans and cattle species as shown in **Table 1**.

Species	Th1 immunity	Th2 immunity	References
Mice	IgG2a	IgG1	[69–72]
Humans	IgG1 and IgG3	IgG4	[73–79]
Cattle	IgG2	IgG1	[18, 80, 81]

Table 1.
Th1- and Th2-associated IgG subtypes in mice, humans, and cattle.

2.4 Cytokines and transcription factors mediate Th1/Th2 cross-regulation

In humans and mice, multiple lines of evidence support that Th1 differentiation inhibits Th2 differentiation, and vice versa [120, 121]. For example, *in vitro* experiments reveal that IFNγ inhibits Th2 differentiation whereas IL-4 suppresses Th1 differentiation [122–124]. In addition, studies using knockout mice and retroviral-transduced CD4+ T cells demonstrate that T-bet blocks Th2 differentiation by inhibiting the transcription of genes associated with Th2 cytokine production [54, 125]. Similarly, GATA-3 prevents Th1 differentiation by suppressing the transcription of genes associated with Th1 cytokines, and interfering with Th1-promoting transcription factors [126, 127]. Collectively, these findings confirm that Th1 and Th2 transcription factors and cytokines cross-regulate each other, ensuring that CD4+ T cells differentiate into either Th1 or Th2 cells. In cattle, however, most of the differentiated clones represent a "hybrid" that co-expresses both IFNγ and IL-4 in the same cell (explained in detail in Section 3) [22, 128]. While it is clear in mice and humans that T-bet and GATA-3 are the transcription factors that regulate expression of IFNγ and IL-4 respectively, at this moment, it is unclear if this is equally true for cattle. In addition, we do not know if the co-production of both Th1 and Th2 cytokines in the hybrid bovine clones corresponds to the co-expression of both transcriptional factors. Therefore, further research is needed to understand the underpinning regulatory mechanism of hybrid clone differentiation in cattle.

2.5 Distinct Th1 and Th2 are the most dominant antigen-specific clones in mice and humans

In mice and humans, Mosmann et al. and Romagnani et al. stimulated single CD4+ T cells *in vitro* and established antigen-specific CD4+ T cell clones, which they classified mostly into Th1 and Th2 subtypes. Although, in both mice and humans, clear-cut Th1 or Th2 were the dominant clones, a small percentage of hybrid clones (named "Th0" clones), that co-produced Th1 and Th2 cytokines (IFNγ and IL-4), were also observed [27, 28]. Subsequently, follow-up research verified the existence of these hybrid clones, which were only a small fraction of the total clones (*i.e.,* only 9.6% clones were Th0) [124, 129–135]. Therefore, at this moment, the consensus in the fields of murine and human immunology is that Th1 and Th2 are the major effector cells that orchestrate immune responses against intracellular and extracellular pathogens, respectively, and that Th0 are short-lived "intermediate" cells [131, 136, 137].

2.6 Th0 is the most dominant antigen-specific clone in cattle

Just a few years after the discovery of the Th1/Th2 subtypes in humans and mice, Brown et al. successfully investigated bovine Th1/Th2 response through the establishment and analysis of antigen-specific CD4+ T cell clones. Peripheral blood mononuclear cells (PBMC) were purified from cattle challenged by experimental pathogens: either intracellular pathogens (*Babesia bovis*, *Babesia bigemina*) or extracellular pathogens (*Fasciola hepatica*) [22]. These purified PBMCs (that contained pathogen-specific CD4+ T cells), were stimulated with antigens derived from the same pathogen used for the challenge, to generate pathogen-specific CD4+ T cell clones, which were then analyzed and classified based on the detection of Th1/Th2 cytokine mRNA. The authors reported that, regardless of the type of pathogen used in the challenge, most bovine clones were Th0 that co-expressed IFNγ and IL-4 (*e.g.,* more than 60% *Babesia* species -specific and more than 90%

Fasciola hepatica-specific clones were Th0) [22]. These observations indicated that bovine Th1/Th2 responses might be at least partially different from the typical murine and human Th1/Th2 responses, as the frequency of bovine Th0 clones was significantly higher than that of murine and humans. Later, when researchers used the Th0 clones specific to an antigen of *Babesia bigemina* to stimulate B cells *in vitro*, both, Th1-related IgG2 and Th2-related IgG1 were detected in the supernatant culture, suggesting that Th0 is capable of performing functions of both Th1 and Th2 cells [138].

3. Many critical bovine pathogens induce Th0 responses

In cattle, mixed Th1/Th2 cytokines (both IFNγ and IL-4) have been detected in cultured PBMCs, or Draining Lymph Nodes (DLNs), or local tissues in large number of diseases. Most researchers commonly refer to this as the bovine Th0 response, which may include clones of all three types (Th1, Th2, and Th0) [128, 139–141]. It is important to note that while Th0 clones can produce both IFN γ and IL-4, Th1 and Th2 clones can only produce a single cytokine, either IFN γ or IL-4 (**Figure 4**). Therefore, a mixed population of Th1, Th2, and Th0 cells possibly contributes to the induction of Th0 responses in most of the bovine diseases as explained in Section 4.

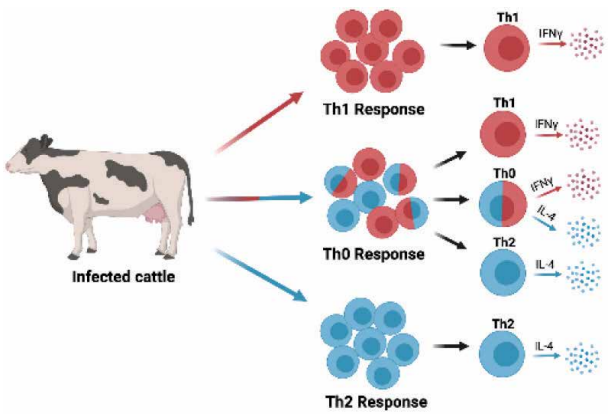

Figure 4.
Helper T cell responses to infections in cattle. Pathogen infections in cattle may induce three types of CD4+ T cell responses: Th1, Th2 and Th0. Th1 responses are characterized by Th1 clones that produce IFNγ, Th2 responses include Th2 clones that produce IL-4, and Th0 response could induce mixed populations of clones: Th1, Th2 and Th0. Th0 clones co-express both IFNγ and IL-4.

4. Advancement of technology facilitates the progress in bovine immunology

Technology is a critical factor that drives the advancement of science, and bovine immunology is not an exception, particularly regarding bovine CD4+ T cell research. In the late 80s, the study of bovine Th1/Th2 responses depended heavily on the measurement of cytokines in the supernatant of cultured CD4+ T cells through simple biological assays such as ELISA, or detection of IgG subtypes in the serum of infected animals through ELISA or immunoblotting techniques. In this context, upregulation of supernatant IFNγ and serum IgG2 would represent a Th1 response, upregulation of IL-4 and detection of serum IgG1 would indicate a Th2 response [18, 80], and detection of both cytokines and both IgG subtypes (IgG1 and

IgG2) would represent a Th0 response [142]. In the late 90s, advancements in molecular biology enabled scientists to measure cytokines at the transcriptional level (mRNA). Thus, reverse transcription polymerase chain reaction (RT-PCR) was commonly used to detect the presence of mRNA of Th1/Th2 cytokines in PBMCs, DLNs, and tissues of infected cattle [143–145]. In the next decade, the advent of quantitative PCR (qPCR) improved the detection of Th1/Th2 transcripts from a qualitative to a quantitative level [146]. Later, with the invention and use of flow cytometry, scientists were able to measure protein production of Th1/Th2 cytokines on a population level [147]. More recently, some very exciting technological advancements have been developed, such as single-cell RNA sequencing, proteomics, metabolomics, confocal microscopy, which are considered excellent tools for a deeper understanding of immune mechanisms [148–152]. Therefore, the advancement of bovine immunology research is closely associated with the development of novel technology in science, especially in the context of understanding Th1/Th2 responses in cattle.

4.1 Most intracellular pathogens induce either a Th1 or Th0 response in cattle

During pathogen invasion, the host mounts a CD4+ T cell response that may or may not be effective enough to clear the infection. In humans, ineffective CD4+ T cell responses are associated with increased pathogenesis and progression towards chronic infections [153]. Cattle mostly launch either Th1 or Th0 responses against intracellular pathogens [154–157]. However, some bovine pathogens are able to establish chronic infections, which is possibly associated with ineffective CD4+ T cell responses [128, 158].

As observed in mice and humans, bovine Th1 responses are considered to be protective against diseases caused by intracellular pathogens such as *Theileria annulata*, and *Anaplasma marginale* [154, 155]. Indeed, researchers in the late 80s found that transferring serum from an immune animal into animals infected with theileriosis was not effective at controlling infection [159]. Several groups later discovered that CD8+ T cell responses but not humoral responses were effective at controlling disease, since antigen-specific CD8+ T cells from recovered animals demonstrated effective cytotoxicity to the autologous infected cells *in vitro* [160–162]. Further research revealed that *in vitro* activation of T cells with *Theileria*-infected macrophages predominantly induced IFNγ expression [163]. Similar to theileriosis, Th1 responses were also protective against *Anaplasma marginale* [155, 164]. In both infected and vaccinated animals, circulatory IFNγ levels were higher relative to their healthy counterparts [155, 164]. Similarly, IgG2 was increased in cattle infected with *Anaplasma marginale* [165]. Collectively, in both theileriosis and anaplasmosis, hosts seem to induce effective Th1 responses.

Bovine pathogens such as *Mycobacterium tuberculosis* and *Mycobacterium paratuberculosis* can shift a Th1-dominant response towards a Th0- or a Th2-dominant response as the infection progressed [128, 158]. In bovine tuberculosis, high levels of circulatory IFNγ are detected at the early stage of disease that could inhibit Mycobacterial growth, suggesting that the host most likely mounts an early Th1 response [166–168]. However, in the chronic tuberculosis increased serum IgG1 (a Th2 associated antibody) is detected in the serum [128]. In line with these observations, in mice and humans, IFNγ expression was upregulated during the early phases of tuberculosis, however, at the chronic phase IL-4 expression was enhanced [169–172]. Collectively, these results suggest that *Mycobacterium tuberculosis* can shift an IFNγ (Th1) dominant response towards an IL-4 (Th0 or Th2) dominant response at the later stages of disease. Interestingly, the frequency of antigen-specific Th0 clones was higher in animals showing severe lung pathology

than in animals having less severe lesions [128]. Therefore, the authors speculated that Th0 clones may play an important role in skewing the immune response from Th1 (IFNγ) response towards Th0 or Th2 (IL-4) response during the progression of infection (**Figure 4**) [128]. As in *Mycobacterium tuberculosis* infections, the immune responses to *Mycobacterium paratuberculosis* switches from Th1 response to Th2 response while the disease progresses from subclinical to clinical stage [158]. In *Mycobacterium paratuberculosis* infections, cattle show high levels of IFNγ in the supernatant of cultured PBMCs and high levels of IFNγ mRNA in the intestinal ileal tissues, suggesting an induction of Th1 response against this pathogen [173, 174]. Importantly, cattle clinically infected with *Mycobacterium paratuberculosis* had significantly lower expression of IFNγ in ileal and caecal lymph nodes compared to cattle at sub-clinical stage of infection [175]. This finding supports the notion that the suppression of the Th1 response at the sub-clinical stage of the disease might have contributed to the progression of disease into the clinical stage. Furthermore, increased antigen-specific IgG1 was detected in animals infected with *Mycobacterium paratuberculosis* at the clinical stage, suggesting a Th2 response [176, 177]. Together, these findings suggest that the shift of an early-induced Th1-dominant response towards a Th0 or Th2-dominant response is associated with disease progression in both bovine tuberculosis and bovine paratuberculosis.

During the early phases of Respiratory syncytial virus (RSV) infection in humans and mice, the host launches a Th1/Th2 mixed response (*i.e.,* both IFNγ and IL-4), which then shifts towards a Th2 response (*i.e.,* increased circulatory IL-4) during chronic infection [178–180]. Consistently, cattle infected with Bovine respiratory syncytial virus (BRSV) seem to mount a Th0 response, which turns into a Th2 response during chronic infection [143, 181]. In the past, reports suggested that both IFNγ and IL-4 were detected in the peripheral blood, lymph sample and pulmonary tissues of BRSV infected animals at the early stage, indicating the induction of a Th0 response [144, 181, 182]. Similarly, both IgG1 and IgG2 were detected in the serum, although they peaked at different times during infection [182]. Conversely, IgE and IgG1 levels increased as the infection progressed towards the chronic stage, suggesting a gradual shift from a Th0 towards a Th2 response [143, 181–183]. Collectively, these studies indicate that these pathogens can switch the early-induced Th0 response towards a Th2 response during chronic infection.

The efficacy of Th0 responses in controlling infections caused by bovine intracellular pathogens is unclear. While Th0 responses seem ineffective against some bovine diseases such as tuberculosis, they can be protective against bovine babesiosis and non-cytopathic Bovine viral diarrhea virus (ncp- BVDV) infection [156, 157, 184]. In Babesiosis, both CD8+ T cell responses and humoral responses appear critical to clear infection. For instance, increased numbers of antigen-specific CD8+ T cells were detected in the peripheral blood of vaccinated animals [156]. Similarly, transferring serum from an immune animal containing both IgG1 and IgG2 can clear infection of sick animals [184]. In this regard, *in vitro* experiments have demonstrated that the majority of Babesia-specific clones are Th0, which are able to stimulate B cells to produce both IgG1 and IgG2 [22, 138, 184]. Furthermore, IgG1 and IgG2 antibodies were found effective to prevent invasion of bovine erythrocytes by *Babesia bovis* merozoite *in vitro* [185]. Collectively, these findings suggest that Th0 responses promote both the cytotoxic activity of CD8+ T cells, and neutralizing activities of IgG subtypes [156].

Cattle might launch different immune responses against different biotypes of the same intracellular pathogen [145, 186, 187]. For instance, while Th0 response was induced against the non-cytopathic (ncp) biotype of Bovine viral diarrhea virus (BVDV), Th1 response was induced during infection caused by the cytopathic biotype (cp) [188]. In experiments with T cells isolated from the ncp-BVDV

Disease	Detected cytokines	Serum antibodies	References
Theileriosis	IFNγ (RT-PCR)	—	[154, 163]
Anaplasmosis	IFNγ (ELISA)	IIgG2 /IgG1 + IIgG2	[155, 189]
Babesiosis	IFNγ + IL-4 (RT-PCR)	—	[22, 138, 156]
Respiratory syndrome	IFNγ+IL-4(flow cytometry)	IgE	[181, 182]
Bovine viral diarrhea	IFN γ / IFNγ + IL-4 (q-RT-PCR)	IgG2/ IgG1 + IgG2	[145, 186, 187]
Tuberculosis	IFNγ to IL-4 shift (PCR)	IgG2 to IgG1 shift	[190]
Paratuberculosis	IFNγ to IL-4 shift (ELISA+ RT-PCR)	IgG2 to IgG1 shift	[158, 191, 192]

Table 2.
Characterization of helper T cell responses in diseases induced by bovine intracellular pathogens. Th1/Th2 cytokines were detected in cultured PBMCs and DLNs; IgG subtype was tested in the serum.

infected cattle, IL-4 protein in the supernatant of CD4+ T cell culture and IFNγ protein in CD8+ T cell culture were detected, suggesting possible induction of Th0 response [157]. More recently, Palomares et al. analyzed cytokine expression in tracheo-bronchial lymph nodes and found that both IFNγ and IL-4 were detected in ncp-BVDV-infected cattle, but IL-12 mRNA was only detected in cp-BVDV-infected cattle [145]. Additionally, while only IgG2 was detected in the serum of cp-BVDV-infected cattle, both IgG1 and IgG2 were detected in ncp-BVDV infected cattle after day 35 of infection [187]. These results collectively reveal that ncp-BVDV induces a Th0 response whereas cp-BVDV induces a Th1 response in infected cattle.

Thus, available literature supports the notion that cattle launch either Th1 or Th0 responses against most infectious diseases caused by intracellular pathogens (**Table 2**). Moreover, although further research is required to confirm these findings, the shift from an early Th1 or Th0 response towards a Th2 response is associated with progression of disease towards chronic condition.

4.2 Most extracellular pathogens induce either a Th2 or Th0 response in cattle

In mice and humans, Th2 responses are typically effective in controlling extracellular pathogens. In this regard, Th2 cytokines can induce processes such as IgG subtype switching and migration of mast and eosinophils to the site of infection that are critical for defending the host against extracellular bacteria and parasites [98]. In cattle, most of extracellular parasites induce either Th2 or Th0 responses [193–195]. However, some pathogens are capable of suppressing Th2 response, which is associated with the establishment of chronic infections [196].

Generally, Th2 responses are effective in controlling gastrointestinal nematodes such as *Cooperia oncophora* [197, 198]. Infected animals had increased level of antigen-specific IgG1 (Th2 associated antibody) in the serum [199]. Consistently, a high titer of pathogen specific IgG1 was associated with a better immune response [200]. Similarly, increased numbers of peripheral eosinophils (a Th2 response feature) was associated with increased expulsion of cooperial larvae [200]. Importantly, cytokine analysis of the intestinal tissue of disease resistant cattle demonstrated high expression level of IL-4 and IL-13 mRNA compared to those susceptible animals [201, 202]. These results offer compelling evidence that Th2 response is critical to control infection caused by some extracellular pathogens such as *Cooperia oncophora*.

Interestingly, some extracellular parasites such as *Dictyocaulus viviparus* (lung worm) are capable of shifting the initial Th2 or Th0 response into an ineffective Th1 response to establish chronic infections [203, 204]. At the early stage, both IL-4 and IFNγ were detected in the lungs and DLNs after day 15 of lung worm infection, indicating an initial Th0 response [205]. However, subsequent research only detected increased IL-4 mRNA for a short period of time in the Broncho-alveolar lavage fluid (BALF) of infected cattle, suggesting a possible Th2 response [206]. In line with this finding, high level of total IgE (antigen-specific plus non-specific) in the serum and BALF was associated with the clearance of lungworm [203]. Furthermore, in the chronically infected animals the detection of Th1 associated antibody (*i.e.,* IgG2) in the serum, was associated with increased lungworm larval excretion [204]. These data indicate that bovine lungworm might shift the early-induced Th0 or Th2 response towards a Th1 dominant response to establish chronic infection.

In cattle *Fasciola hepatica* (liver fluke) can modulate the early-induced Th1 or Th0 response into an ineffective Th2 response at the later phases of the disease [207, 208]. Of note, although an initial Th1 response was observed in the peripheral blood, a Th0 response was also observed inside the hepatic lymph node, as indicated by the detection of both IFNγ and IL-4 [209–212]. Collectively, these experiments suggest that cattle might launch either a Th1 or a Th0 response at the early stages of liver fluke infection. However, at later stages, the response is shifted to a Th2 response as indicated by the significantly increased expression of IL-4 mRNA (x6) and significantly reduced expression of IFNγ mRNA (x6) in the hepatic tissue of infected animals, which is consistent with several other reports [140, 213, 214]. In line with these observations, peripheral blood lymphocytes obtained from chronically infected animals failed to induce IFNγ secretion when co-cultured with adult fluke antigen *in vitro* [209]. Importantly, chronically infected cattle typically show high levels of antigen-specific IgG1 in the serum [140]. Altogether, these findings suggest that *Fasciola hepatica* might switch a Th1 or a Th0 dominant response to a Th2 dominant response at the chronic stage of disease.

Ostertagia ostertagi (OO), an economically important abomasal nematode, typically induces Th0 response [215]. Bovine OO usually causes chronic infection and requires long-term repetitive exposure (at least 2 years) to develop effective immunity [216]. Both pathogen-specific IgG subtypes (IgG1 and IgG2) were detected in OO-infected cattle, with higher serum IgG1 titer than IgG2 [217]. Similarly, mRNAs of both IL-4 and IFNγ were upregulated in the abomasal lymph nodes of experimentally infected cattle from day 11 to day 28 after infection, suggesting the induction of a Th0 responses [215]. In contrast to this observation, subsequent research demonstrated induction of Th2 response in the abomasal lymph nodes of OO infected cattle [218]. The differences observed between these two experiments might be explained, at least in part, by the differences in time points for cytokine detection and in the number of L3 larvae used for experimental infection. More specifically, while Canals et al. measured cytokine expression from day 11 to day 28 post infection and used 200,000 L3 larvae for experimental infection, Claerebout (2005) measured cytokine expression after 8 weeks post primary infection and only used 25,000 L3 larvae [215, 219]. Recently, Mihi et al. experimentally infected cattle with 200,000 L3 larvae and tested the gene expression of Th1/Th2 cytokines at different time points; interestingly, the authors observed a positive association between upregulation of both IFNγ and IL-4 (in mucosa) with migration of adult (L5) worms out of gastric gland towards abomasal mucosa [146]. These observations suggest that *Ostertagia ostertagi* may modulate the bovine immune response by inducing a Th0 response, which is ineffective in controlling OO and leads to the establishment of chronic infections.

Disease	Detected cytokines	Serum antibodies	References
Cooperiosis	IL-4 (q-PCR)	IgG1	[201, 202, 227]
Lung worm infection	IL-4 /IL-4+ IFNγ (RT-PCR)	IgG1, /IgG1 + IgG2	[228, 229]
Trichomoniasis	—	IgG1 + IgG2	[221, 222, 230]
Fasciolosis	IL-4 (ELISA+ qPCR)	IgG1	[211, 231, 232]
Ostertagiasis	IL-4 + IFNγ (qPCR/RT-PCR)	IgG1 + IgG2	[146, 215, 218]

Table 3.
Characterization of helper T cell responses in diseases caused by bovine extracellular pathogens. Th1/Th2 cytokines were detected in cultured PBMCs and DLNs; IgG subtype was tested in the serum.

Immune response against extracellular pathogens may vary at the systemic and local levels, such as in bovine trichomoniasis, where Th0 response is induced in the serum, and Th2 response in the mucosal secretion [220, 221]. More specifically, *Trichomonas foetus* upregulates both IgG1 and IgG2 in the serum but only IgG1 in local secretions from cervix, vagina, and uterus [220, 221]. Furthermore, animals immunized with specific antigen of *Trichomonas foetus* showed resistance to the experimental challenge, which was associated with the upregulation of both antigen-specific IgG1 and IgG2 in the serum [222, 223]. *Trichomonas foetus* seems to induce a Th0 response in the circulation, but a Th2 response in the mucosa. In addition, the systemic Th0 response may be protective against *Trichomonas foetus* rechallange.

Generally, Th2 response is effective in controlling extracellular bacteria [224]. For instance, Th2 response controls *Clostridium difficile* infection *in* humans *and Streptococcus suis* infection in pigs [224, 225]. In cattle, only few reports are available on CD4+ T cell response to extracellular bacteria such as *E. coli*. At this moment, the common understanding is both CD8+ T cell and antibodies seem to be critical to generate protective immunity (consistent with humans) in *E. coli* 0157:H7 infection [193, 194, 226].

Collectively, the results obtained from multiple experiments indicates that extracellular pathogens typically trigger Th2 or Th0 responses in cattle as shown in **Table 3**, and some extracellular pathogens modulate initial Th2 or Th0 responses to ineffective Th1 responses that are associated with the development of chronic infection.

4.3 Pathogens regulate the availability and the strength of three critical signals to suppress effective CD4+ T cell responses

Whenever a pathogen invades and starts multiplying, the host mounts a coordinated attack in order to clear the infection. To counteract the host attacks, some pathogens can interfere with helper T cell responses to establish chronic infections. This can be achieved through unique strategies that impair the availability or strength of the signals required for the activation and differentiation of CD4+ T cells (**Figure 1**). For example, pathogens such as *Salmonella*, and *Mycobacterium tuberculosis* can downregulate MHC-II expression in APC, which diminishes the strength of the 1st signal (antigen stimulation) [233, 234]. In addition, pathogens can reduce the expression of co-stimulatory molecules (2nd signal) and change the type of APCs (*e.g.* dendritic cell vs. macrophage), which

can collectively impair all of the three signals required for the activation and differentiation of T cells (**Figure 1**) [235, 236].

Bovine pathogens escape from effective CD4+ T cell responses in a very similar way to those of mice and humans. They can regulate the availability, type, and strength of three signals. Some pathogens such as Bovine herpes virus type-1 (BHV-1), *Bovine papilloma virus (BPV)*, and *Mycobacterium paratuberculosis* can undermine the strength of antigen stimulation (1st signal) by downregulating MHC-I expression, which is actively involved in antigen presentation to CD8+ T cells [237–239]. Similarly, some pathogens can disrupt the host T cell response through inhibiting the co-stimulatory signals [211, 240, 241]. Co-stimulatory molecules expressed on the surface of CD4+ T cells (as shown in **Figure 1**) are of two types: one provides activating signals, and the other provides inhibiting signals [242]. Pathogens such as, *Bovine leukemia virus*, *Anaplasma marginale*, and *Fasciola hepatica* can upregulate the expression of inhibitory molecules like program cell death protein-1 (PD-1), which severely impairs the T cell response when these inhibitory molecules bind to their ligands on the surface of APCs [211, 240, 241]. Additionally, pathogens such as *Ostertagia ostertagi* and *Myctobacterium paratuberculosis* can induce immune-regulatory cytokines that can inhibit the activation, differentiation, and expansion of effector CD4+ T cell subtypes [26, 243, 244]. More specifically, *Ostertagia ostertagi* may stimulate neutrophils to produce IL-10, which can suppress bovine CD4+ T cell activation [26]. Furthermore, pathogens like *Fasciola hepatica* can reduce the number of APCs by apoptosis, which curtails the availability of all activating signals [211]. Moreover, pathogens such as *Anaplasma marginale, Bovine herpes virus – 1* and *Bovine viral diarrhea virus* can directly cause apoptosis of antigen-specific CD4+ T cell and starkly compromise the ability of the host to co-ordinate effective CD8+ T and antibody responses [241, 245–247]. In short, bovine pathogens regulate the CD4+ T cell responses by reducing the availability and strength of the three activating signals by changing the type and number of APCs, or by interfering with co-stimulation and cytokine production.

4.4 Pathogens regulate the CD4+ T cell differentiation process to establish chronic infections in cattle

In addition to regulating activation signals, during the course of infection, pathogens can also regulate CD4+ T cell differentiation to evade the effective immune response mounted by the host. As already explained, intracellular pathogens can shift effective Th1 response to an ineffective Th2 response; similarly, extracellular pathogens can shift an effective Th2 response to an ineffective Th1 response, in order to promote the chronic infection in the host. For example, *S. japonicum* in mice can shift a Th2 response to an ineffective Th1 response by triggering apoptosis of Th2 cells via granzyme B signal pathway [248]. Similarly, some authors suggested that in chronic diseases such as bovine tuberculosis, immune complexes circulating in the blood might interfere specifically with Th1 response thus leading to a relatively increased Th2 response [249]. In cattle, intracellular pathogens including *Mycobacterium tuberculosis, Mycobacterium paratuberculosis and Bovine respiratory syncytial virus* (BRSV) shift the immune responses from a Th1 or a Th0 to an ineffective Th2 response, to establish chronic infections [128, 158]. In the same manner, extracellular pathogens such as *Dictyocaulus viviparus* modulate the immune response from a Th0 or a Th2 response to an ineffective Th1 response and establish the chronic infection [203, 204]. In summary, a fraction of bovine pathogens can skew the CD4+ T cell polarization to an ineffective subtype that cannot control their infection, which leads to the establishment of chronic infections.

5. Conclusion and future directions

After receiving three stimulation signals from APCs, naïve CD4+ T cells differentiate into effector subtypes such as Th1, Th2, and Th0 cells. While clear-cut Th1 and Th2 are the common subtypes detected in mice and humans, hybrid Th0 is common in cattle infected by both intracellular and extracellular pathogens. In fact, Th0 responses induced in many bovine diseases might consist of a mixed population of Th1, Th2, and Th0 subtypes. Thus, despite similarities in general, bovine CD4+ T cell responses seem to be partially different from the Th1/Th2 responses classically defined in mice and humans. Therefore, understanding the mechanisms of bovine CD4+ T cell differentiation and its regulation by pathogens may facilitate the development of more effective vaccines and designing immune intervention strategies against important chronic bovine infectious diseases.

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Conflict of interest

The authors state no conflict of interests.

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Chapter 8

Adverse Impact of Heat Stress on Bovine Development: Causes and Strategies for Mitigation

Golden Gokhale and Guru Dutt Sharma

Abstract

Heat stress induces the richness and reproductive domesticated animal's performance by settling the physiology conceptive steps, through hormonal irregularity, diminished oocyte quality and feeble semen quality, and diminished undeveloped organism advancement and endurance. It depends on principally milk production, nutrition, disease management, sexual activities, and heat stress tolerance capacity in livestock farming. The decreases infertility caused by elevated blood heat influences sex gland regulation, oestrus regulation, and gametocyte disturbance and also affects embryonic development. Heat stress reduces the degree of dominance of the seminal vesicles and this may be observed as reduced steroidogenic capability of its theca and granulose cells as fall in blood oestrogen concentrations. Plasma progestin levels are also diminished counting on whether or not the heat stress is acute and on the metabolic state of the animal. The endocrine changes the cyst activities and alters the ovulatory mechanism leading to a decrease in gametocyte and embryo quality. Summer infertility may be countered through oestrus behaviour can be mitigated by with the help of implementation of ovulation phase treatments to limited period of embryonic transfer and also advanced reproductive technologies involving hormonal treatments, systematic artificial insemination and which may enhance the possibility of establishing pregnancy in domestic animals.

Keywords: Heat stress, livestock, Mitigation, Infertility, Reproductive

1. Introduction

Global warming has multifaceted consequences for livestock today, which exhibits as heat stress, lack of feed and fodder, and alters in epidemiological patterns of vector borne diseases among other things resulting in decline in reproduction performance in production. In Dairy and Beef industries heat stress is the major cause for production loss. Bovines are homoeothermic organisms sustain a balanced body temperature by balancing the quantity of metabolic activities generated heat and also the heat depletion to the surroundings [1]. The heat development and loss keep body temperatures in a narrow range, but illness, inadequate nutrition, and extreme environmental temperatures can disturb the metabolism.

At the time of heat stress, animal productivity and reproduction output reduced dramatically. It also declines the rate of food consumption, milk production, dairy cow health, and reproduction. The upper crucial limit of thermo-neutral zone cattle

is approximately 25°C. When the temperature exceeded above the 25°c denotes that dairy cattle can get affected by heat stress [2]. In Lactating cattle's are further vulnerable to heat stress for that reason lactation causes high metabolic energy production, which can lead to hyperthermia. Heat stress appears to have an impact on fertility in the autumn [3]. The lack of fertility, usually related with the June, September, October and November. Thus, cattle are no longer being affected to heat stress [3]. The antral follicle may get affected by the heat stress for long term which further developed 40th to 50th days later into an enlarged dominant follicle [3]. The oocyte get effected by heat stress at the time of pre-ovulatory cycle with involvement of oxidative stress detected in vivo and vitro studies [4, 5]. The administration of antioxidants reduced heat shock in vitro [5]. The embryo pre-implantation is vulnerable towards heat stress, but this vulnerability show reduction in developing embryo. The state of energy balance in lactating cattle's influenced the pattern of follicular growth as well [6]. The negative energy balance and their factors affect the lactation phase, milk productivity level, intake of calcium salts of long-chain fatty acids in energy-rich nutrients and vaccination of bovine somatrophin under the pressure of follicular dynamics. The post-partum lactating and non-lactating cattle's have different numbers of large follicles and E2 ratios during the pre-ovulatory phase. The Temperature-Humidity Index (THI) is a commonly used environmental assessment index for assessing heat stress in dairy production [7]. The values of THI can be categorised into four groups based on the degree of heat stress faced by dairy cattle's. According to Armstrong [2] normal heat stress (71), middle heat stress up to (72 to 79) moderate heat stress (80 to 90), and harsh heat stress (> 90). In tropical and subtropical climates, the THI 72 level is the threshold for high output in terms of lactating and reproduction. The recent studies on THI in temperate climates, on have found that a THI of less than 68 is appropriate for cattle efficiency and welfare.

Heat stress can described with the help of temperature-humidity index (THI) reading with the purpose of is consistently above the thermo-neutral region and has a negative impact on a cattle's efficiency. Thus, the THI > 72 has been linked to heat stress in beef cattle [8] while THI 75 has been linked to heat stress in bulls [8]. Since THI does not account for exposure to radiation or wind velocity, it may underestimate climatic stress in beef cattle.

The objective of this chapter is to describe what could be known about the mitigated strategies for following to overcome heat stress which impairs embryo development and to address physiological, genetic and environmental problems and to enhance bovine production in hot weather.

2. Heat stress imbalances reproductive hormones

The ovarian functions are controlled by gonadotropin hormone (GnRH) which are secreted from the hypothalamus which help in the activation of pituitary gland which further secrete the luteinizing hormones (LH), follicle stimulating hormone (FSH) and gonadotropin hormones [2]. The impact of heat stress on peripheral blood Luteinizing hormone yet to be determined, as some studies have found an increase, decrease or even no effect [2] of heat stress on LH. The lack of LH levels can also disturbs the secretion of estradiol from dominant follicle which causes greater impact on oestrus cycle, maturation of follicles and also decreases the ovarian functions [2]. However, estradiol is essential for ovarian follicle growth, oocyte maturation, and endometrial proliferation. Furthermore, FSH and LH [9], insulin-like growth factor (IGF), LH [9] and anti-Mullerian hormone (AMH) LH [9] have distinct receptors in granulosa cells (GCs) any disruption in GC quality or proliferation capacity can

have an indirect impact on follicle growth, disrupting oocyte maturation resulting in impaired embryo development and an unsatisfactory pregnancy outcome LH [9]. Heat stress reduces plasma estradiol concentrations in dairy cattle's [10] which is consistent with lower luteinizing hormone (LH) concentrations and reduced follicle dominance, though this outcomes has not always been seen [10]. There is also widespread consensus that FSH secretion increases in the summer, owing to reduced inhibit secretion from small follicles. When a stressor affect the hypothalamic–pituitary–adrenal axis (HPA) which are responsible to stimulate the hormone such as gonadotropin releasing hormone (GnRH), vasopressin, releasing hormone (CRH) and glucocorticoids [11]. while progesterone, gonadotropins, prolactin, and glucagon rises [11]. Furthermore, glucocorticoids minimise the vulnerability of target tissues to sex steroids by inhibiting pituitary development of gonadal steroids. The rapid initial release of LH is induced by arachidonic acid and its metabolites, whereas the prolonged release of LH is mediated by protein kinase C-dependent mechanisms. By inhibiting the hydrolysis of phospholipids and thus, preventing the synthesis of arachidonic acid, glucocorticoids reduces the release of LH. Gonadal steroids also have ability to control pituitary gonadotropin activity is also influenced by glucocorticoids [11]. The amount of gonadal steroid hormones will decline in the presence of glucocorticoids over hours or even days [11] disrupting reproductive physiology, behaviour and lowering feeding and appetite [11].

3. Alteration in the mechanism of female reproduction by heat stress

Cattles reported an elevated occurrence of early embryo development during warm seasons for a variety of reasons. Heat stress leads to may adverse conditions at various phases of female reproduction (**Figure 1**). The direct impact of heat stress on oocyte competence and follicular is one of the major cause [2]. Furthermore, adverse impact of heat stress on cattle's super ovulation response, as well as the number and quality of recovered embryos [2]. The heat stress also reduces weight along with diameter of the corpus luteum, as well as the amount of progesterone it releases and the consistency of the oocytes, both contributes to pregnancy loss. The heat stress changes the endometrial environment by up regulating glycoprotein 2 and neurotensin, which can lead to infertility during the summer [11]. All of these reasons decrease the rate of fertilisation and the quality of any resulting embryos, raising the risk of pregnancy failure and lowering reproductive success. On the other hand, beef cattle [11] and dairy cattle have both shown this behaviour. The rate of conception in lactating cattle's decreases as the strength of the heat stress increases. The heat stress event can also affect conception rates from the month before breeding to two weeks after breeding [12]. In addition to these, heat stress is also linked to a smaller concepts scale, which could affect maternal pregnancy recognition and corpus luteum function [12]. Furthermore, heat stress has been linked to a compromised pregnancy during the pre-implantation phase, with an increased risk of foetal loss between days 21 to 30 of pregnancy [12]. The lack of blood flow in uterine can also show impact on nutrients supplementation to embryo and also lesser the secretion of hormones of uterus [12], can further have complicate things. The lactation of dairy cow is primarily effected by heat stress. During heat stress, non-lactating dairy animals and beef cattle are far less likely to become infertile. The conception rates in Holsteins decreased in the summer in Florida for lactating cattle's but not for non-lactating heifers [13]. However, the large amount of quantity of heat emission can process to lactation in the cattle's but the lactating cow is particularly vulnerable to heat stress. The lactation in cattle's will experience hyperthermia (high body temperature) at temperatures as low as 77-284°F [13].

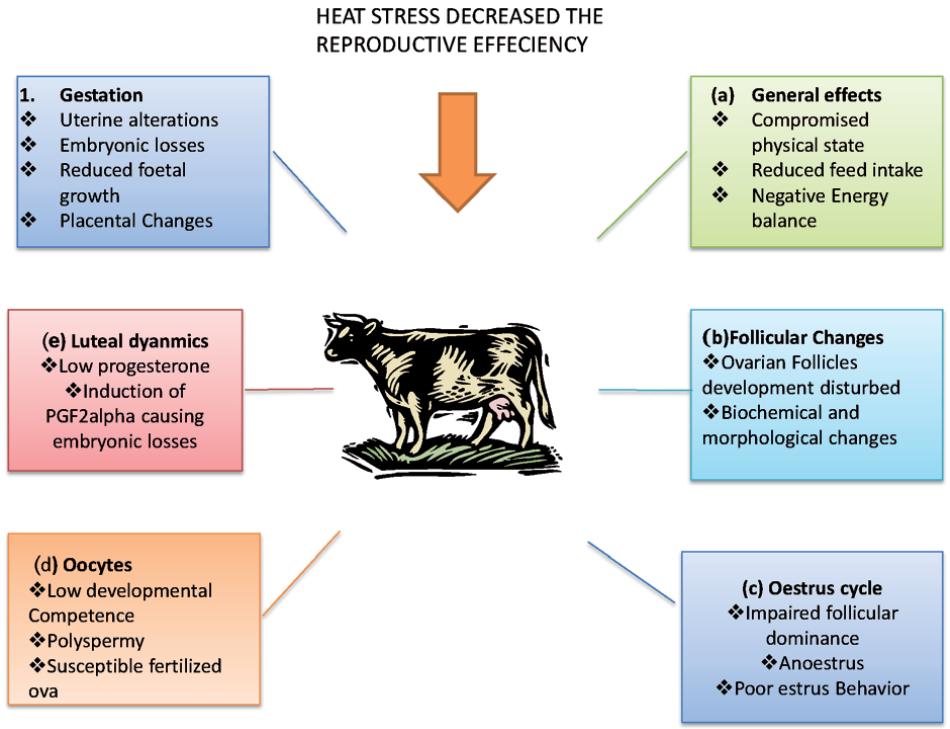

Figure 1.
Impact of heat stress at various phase of female reproduction.

3.1 Heat stress impact on oocyte development at different stages

3.1.1 Oestrus expression

The time interval and potency of oestrus are also abridged by heat stress. Heat stress reduced follicular estradiol, which may have lowered the level of oestrus. The physical inactivity brought in by heat stress may also be a factor in decreased oestrus speech. During oestrus, cattle's become less active hence, less likely be carried out to other cattle's. As a result, dairy cattle's in the summer had about half as many mounts per oestrus as dairy cattle's in the winter. It also diminished the oestrus activity as a result, the Cattles decreased motor activity, which is an attempt to minimise endogenous heat production. In mammalian oestrus also serves as a behavioural predictor, indicating whether or not the female is bred close to ovulation due to a climatic variation shows 80% impact on estruses which are failed to be identified in heat-stressed cattle's [9]. The long period of time towards high temperature decline the rate of pregnancy by shortening the oestrus signs and lowering their severity of pregnancy [9]. Furthermore, during the summer, hot weather triggers ovulation without any symptoms of oestrus [9]. The key cause of imbalanced heat detection represents lower intensity of E2 in blood which alter the steroid genic mechanism of heat stress which is disturbed by granulose cell (GCs) [14]. The increased in rectal temperature seemed to have less effective towards binding protein such as insulin like growth factor, level of progesterone in dominant follicle and in E2 [14]. These various responses must be taken into account in terms of exposure period, oestrus cycle, nutritional quality [14] and other environmental factors including wind and humidity. Since lactation in cattle's produce of high heat can result in milk productivity and ovarian function in lactating cattle's differs from dry cattle's and heifers [14].

3.1.2 Follicular development and oocyte quality

The phase of ovarian folliculogenesis takes about 180 days. At birth, the ovary contains primordial follicles containing an oocyte [5]. The accumulations of primordial follicles in the ovarian pool result in rising of follicles enhance the follicular dynamics and at last increase in the pre-ovulatory follicle. The early changes in inhibit, estradiol, and progesterone have been shown to stifle the growth of primordial follicles. The heat stress imbalance the development of intermediate-size (6-9 m) follicles as result in earlier emergence and sometimes decrease or delayed in dairy cattle's [5]. The possibilities of folliculogenesis are reduced due to which considerable amount of plasma inhibin secreted by small size and intermediate follicles [4]. The earlier development of the pre-ovulatory follicle and a rise in the period of dominance [4] are also correlated with a lower rate of conception [4]. However, follicles produce oestrogen; a hormone that causes cattle's to become overheated. Since smaller follicles contain less oestrogen than larger ones, oestrus activity will be reduced. The oocytes and somatic cells that synthesise estradiol are originate from ovarian follicles whereas estradiol has a number of functions, including inducing of oestrus and cause the LH surge. Heat stress disturbs the follicle range and lengthens follicular waves, lowering oocyte output. It also allows for the development of multiple dominant follicles, which explains why cattle's conceiving in summers has more twins. Heat stress also damages the somatic cells (theca and granulosa cells) within the follicles. The variations in folliculogenesis patterns are likely to accompany changes in oocyte quality caused by heat stress. In cattle's exposed to heat stress, follicular dominance is reduced, ensuing in a boost large number of large follicles on the ovary. Over a longer period of time of ovulatory follicle dominance can enhance the higher secretion follicle stimulating hormone (FSH) and decrease the secretion of estradiol-17 hormone and inhibin hormone [13]. The heat stress in dairy cattle's reduced estradiol production and granulosa cell viability, as well as androstenedione production by thecal cells [15]. The conditions of follicles beneath heat stress is forced by the metabolic markers as result disturbs the level of glucose in blood and also imbalances the levels of non-esterified fatty acid (NEFA). In the cool season, the level of glucose in bovine follicular fluid is around 85% of the level of plasma glucose, and in the hot seasons, the follicular glucose level falls substantially with a corresponding drop in blood glucose level [15]. On the other hand, heat stress does not show any impact on balance of Non-esterified fatty acid despite a substantial increase in plasma levels [15]. The studies suggested that at the time of summer, the conditions of follicles is effected by level of nutrition in blood and level of components of biochemical in body of cattle. However, the concentration of oxygen in fluid of follicular does not get disturbed by heat and non-stressed conditions [15].

3.1.3 Corpus luteum

The progesterone, which is required for embryonic growth, is secreted by the Corpus luteum. The lack of luteal deficiency describes the condition of corpus Luteum that does not secrete sufficient amount of progesterone to maintain the pregnancy as result decrease in the fertility of cattle's. When cattle's are subjected to long-term, persistent, seasonal heat stress, their progesterone levels normally drop significantly [16]. These are may be due to a disturbance in the Corpus luteum formation process, low synthesis of under hyperthermia, or imbalanced pre-ovulatory follicles that shape a Corpus luteum with suboptimal purpose [16]. The lack of progesterone has been observed in at the of summer in luteinized granulose cell and theca cell compared to winter. However, a less significant corpus luteum

and lesser progesterone plasma concentration in the later dioestrus result from a smaller diameter and less steroid concentration in pre-ovulatory follicles, which can compromise embryo implantation and development [13].

However, future studies may require a large number of animals to concluded the comparatively weak consequence of acute heat stress during the delayed follicular processes.

4. Effect of heat stress on oocyte function and development competence

The bovine oocytes are extremely compromised of heat stress impact in cattle's [1]. The high temperature affect germinal vesicle (GV) maturation phase of oocyte which shows vulnerability in bovine oocyte. Heat stress affects the developmental competence of germinal vesicles in oocytes of Holstein cattle's, as evidenced a decrease in consequent embryonic development [1] demonstrated that exposing. The Holstein to environmental chamber at heat stressed are 42°C for 10 hours for done successfully. The number of Normal embryos was reduced during oocyte maturation as compared to control (24°c) [1]. The three successive oestrus cycles can be required for an oocyte to recover from heat stress and then recover its oocyte competency in the following for season [9]. Over a long period of time, heat stress can cause major impact on ovarian pool of oocytes. The impact might also disturbed cow fertility and harmed even in the autumn (when there is no ambient thermal stress). As demonstrated by the oocyte competence stability and pregnancy in warmth weather which follow in winter. The hyperthermia of maternal half of concentration of ovarian follicles but does not affect the follicular pool. The oocyte maturation failure can take a variety of forms when exposed to heat stress. It interferes with the biosynthesis of steroid hormones, which are responsible in oocyte maturation regulation mechanisms [9].

5. Different cellular alteration induced by the heat stress in bovine oocyte

The processes by which impact of high temperature affect physiology of oocyte is still not identified but some studies consider that it has been identified that high temperature can damage the cell structures and organelles depending on the basis of temperature [1]. In biological membranes, cytoplasmic and nuclear compartments, heat induce can cause cellular damage in bovine oocytes had been observed. However, evidence suggests that the cytoplasm of the oocyte is more vulnerable towards negative effects of high temperatures as compared to the nucleus [1]. Heat stress may also prevent oocytes from maturing their nuclei, resulting in a decline in polar body charge. Heat stress perhaps, can cause oocytes cytoskeletal structure to be disrupted [9]. In the cumulus oophorus complex however, heat stress controls the appearance of HSP70, the apoptotic gene caspase-3 and other antioxidant superoxide dismutase (SOD1), catalyse (CAT), and Complexin (CPX4). These changes in gene regulation resemble coefficient of coincidence (COC) self-defence mechanisms when they are exposed to heat stress. The 70 kilo Dalton heat shock proteins (HSP70) is a multiple effect factor that keeps the intracellular surroundings are stable and decline cell death [9]. By controlling Caspase-3 and cytochrome c, HSP70 expression can also defend cells from apoptosis [9]. The higher level of HSP70 appears to aid oocyte survival from heat stress by up regulating SPKH1, BCL-2, SOD1, CAT, and CPX4 while down adaptable p53. The changes can be brought by heat shock in bovine oocytes because of lack of nuclear maturation. The oocytes that entered metaphase II phase (MII) following In vitro maturation (IVM) was decreased germinal vesicle (GV) stage [1] and maturation

of oocytes [1]. By growing the amount of metaphase I (MI) in oocytes. Heat shock stopped meiotic progression. On the other hand, in one study it is demonstrated that heat shock at 41°c after 16 to 18 hours it increases the maturation of nuclear and also increasing amount of MII oocytes [1]. The high temperature can cause cellular harm in oocytes of bovine observed in the cellular compartment along with region of cytoplasmic and in nucleus. The cytoskeleton of oocyte gets affected when it comes in contact of high temperature. The Heat-induced shows interruption of microtubules and microfilaments affects chromosome segregation at the time of fertilisation and cell division, as well as the division of cellular structures like cortical granules and mitochondria. On the other hand, heat shock also causes affect to DNA fragmentation and lowers mitochondrial activity in bovine oocytes, implying that the heat-induced mitochondrial apoptotic pathway is activated for combat the low fertility caused by heat stress, a variety of methods have been used. In bovine oocytes, molecules like IGF-I, caspase inhibitors, and the sphingolipid (S1P) have recently been identified as thermoprotective factors. This factors improved oocyte developmental competence and rescued many cellular functions that had impaired by high temperatures. As a result, identifying and characterisation of cellular thermoprotective molecules may be a viable option for reducing the impact of elevated temperature on reproductive function.

6. Alteration in mechanism of male reproduction by heat stress

The Bulls make up short of the herd, and their reproduction is straightly linked to the fertilisation of oocytes. In order to generate health of bull viability, and genetically modified potential ideas in bulls genes. In mammal species have unique physiological regulation known as thermoregulation that protect the reproductive activities from the climatic circumstances. The testicular temperature for spermatogenesis in bulls cannot go above 33–34°c [14] through spermatogonia to elongated spermatids, spermatogenesis mechanism of bovine is multifaceted which take 61 days to complete their process [17]. However, the timing of spermatozoa exposure to heat stress, as well as the time and withdrawal from heat stress. It is difficult to differentiate that at which phase of spermatogenesis the spermatozoa get affected by heat stress. The collecting ejaculate at the time of spermatogenesis phase denotes that heat stress disturbs the spermatogenesis cycle. Inefficient histone replacement by protamine's, resulting in sperm chromatin conformation shifts, is the cause of cell vulnerability at certain particular periods [17]. Hyperthermia has a detrimental impact on testicular function. The adverse impact of heat stress on reproductive tissue as show impact such as lack of germ cell, low morphology, lack of sperm count. According to requirements of specific cell the DNA of sperm nucleus arranged on basis of requirements. The specific type nuclear protein present in spermatozoon's which sets chromatin in more condensed structure between 6 to 20 times as compared to nucleosome-bound DNA, nucleus [17]. During spermatogenesis, heat stress alter the conformation of chromatin of sperm which further imbalance the conformation of DNA methylation which further represent in reorganisation of zygote [17]. The high compaction is due to the substitution of histone-bounded with chromatin with protamine-bounded with chromatin, which is needed for the secure liberation of DNA of sperm of oocyte in the female reproductive tract under the depletion of oxidative stress. Furthermore, the tremendously condensed sperm nucleus inhibits sperm DNA transcription. Protamine deficiency in the sperm nucleus causes DNA damage, which can result in male sub-fertility or infertility [17]. The heat stress increase the level of thiobarbituric acid reactive substance (TBARs) and also level of oxidative marker vice versa decreasing the level of

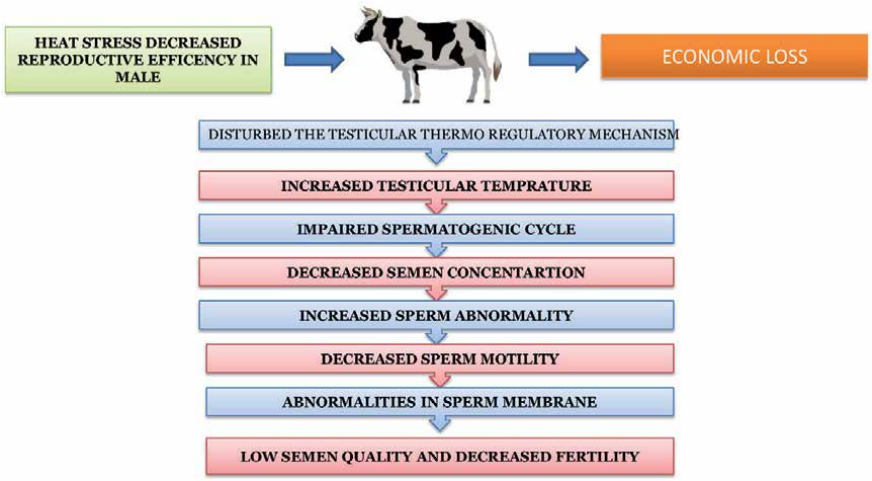

Figure 2.
Impact of heat stress on reproductive efficiency of male reproduction.

glutathione peroxidise (GPx) and enzyme related to antioxidant in seminal plasma of bovines [14]. Bulls plasma luteinizing hormone (LH) levels are reduced by heat stress [14]. The reproductive efficiency of males gets majorly reduced by the impact of heat stress (**Figure 2**).

7. Impact of heatstress on embryo development

The impact of heat stress can cause major impact on maturation of oocyte and development of oocyte competence. The fertilised females under the influence heat stress decline the embryo quality in cattle's [14]. During the fertilisation and implantation of embryo experience towards following phases such as cell proliferation, alteration in patterns of gene expression and cell differentiation. The heat stress significantly inhibits embryo growth 48–72 hours after fertilisation, which correspond to the 8–16 cell stage [14]. After this point, heat stress shows less impact on development and so on cell proliferation [14]. The embryos shows especially vulnerable to maternal heat stress in earliest phase of development as further as development processes the sensitivity towards heat stress. The proportion at which maturation of embryo take place to blastocyst stage after the 8th day of oestrus was decline. When lactating of cattle's were demonstrated on day 1 heat stress impact on oestrus (1-2 cell stage embryo). However, heat stress does not show any impact on blastocyst phase on eighth day [2].

The zygotic genome activation (ZGA) phase occurs at the 4th to 8th cell stage in cattle's [14] is the most vulnerable to heat stress in cow embryos. After the zygotic genome activation heat stress alter the conformation structure of chromatin of embryonic cell [14] potentially disrupting gene expression. Thus, heat stress causes apoptosis in embryonic cells in cattle's [14], and rabbits in addition to maturing oocytes [14].

8. Mitigation strategies for reduction of heat stress

The heat stress impact on cattle's result in considerable financial losses as well as expensive for farmers, but there are following reduction method for heat stress to recoup any of these losses by implementing appropriate heat stress mitigation

strategies. These techniques may be used individually or in combination to improve outcomes by ensuring the best possible atmosphere for farm animals to working farmers are more likely to follow policies that are both cost-effective and incorporate indigenous expertise. Environmental adjustment has traditionally been used to reduce heat load, with the focus on (i) minimising sunlight (ii) increasing air movement [12]. On the other hand wetting of cattle has been subject of research [12] observed that the causes of cooling in day and night, the utilisation of movement of air and water, management of heat load can be measured with help of changes in rectal temperature, respiration time and DMI. There many availabilities of mitigation option for farmers and producers such as (I) oestrus detection (ii) nutritional management (iii) genetic manipulation (iv) antioxidant (v) pharmaceutical treatment (vi) adaptation and acclimation (vii) embryo transfer. In light of antibiotic resistance, nutritional methods are becoming more common. In science also genetics is well known responsible for thermo tolerance capacity, gene identification in cattle's for making them cope up with heat resistance is a new area of research. Individual livestock systems must be assessed for mitigation opportunities to ensure that the mitigation techniques put in place turn into an efficient method for dropping the impact of heat stress in that venture.

8.1 Detection of oestrus and injection at oestrus

Due to the extreme shorter length and lower strength of oestrus, it is difficult to detect. Using a variety of heat detection methods, such as tail-head-paint combined with visual oestrus detection, podometer, pressure enabled patches, and electronic devices put on the tail and head, may boost dairy cow reproductive efficiency. The detection rate for oestrus can be improved by rising the time and several number of visual study [18]. At the time of summer, using an entire male to detect heat at night and early in the morning can improve detection performance [18]. This method suppresses heat stress of oestrus might be hormonal as suggest that indicates heat stress decreases the level of estradiol-17 levels and vice versa increase the secretion level of adrenocorticotrophic which can protect oestrus conduction under the influence of estradiol-induced. The physical lethargy exhibited by heat-stressed cattle's is also likely to reduce oestrus. The other method used for increase fertility in the summer the injection of GnRH are inject during oestrus. According to some studies, when lactating cattle's were vaccinated with GnRH at observed oestrus during the summer, the conception rate increased from 18–29%. The cattle's which are lactating dairy cattle's given GnRH injections at the first indication of status heat during the summer and autumn months had higher conception rates (56%) than untreated (41%) monitors [18]. Heat stress decline the time duration and harshness of oestrus, which leads to an increase in anoestrus and silent ovulation. In order to increase fertility, the timed artificial insemination (TAI) protocol is used for effective oestrus detection and timely insemination. The hormonal therapies have been designed to synchronise ovulation times, allowing for the use of fixed TAI without the need for oestrus detection. The TAI protocol is known as ovary synchronisation content of insemination of gonadotropin-releasing hormone (GnRH) (day 0), prostaglandin F2 (day 7) and GnRH (day 9) and a hormonal therapies, followed by artificial insemination 16-20 hours after the second GnRH hormonal treatment [19]. When coupled with TAI, the ovary synchronisation protocol can successfully synchronise ovulation in cattle's can also enhance conception rate [19]. Under subtropical environmental conditions, the Centre for Inherited Disease Research (CIDR) synchronisation and Pre-synchronisation protocols are also used to increase the rate of conception and rate of pregnancy of Holstein cattle's [19]. This TAI protocol has the potential to minimise reproductive efficiency losses in cattle due to poor oestrus detection in the summer.

8.2 Genetic manipulation

The definite genes that regulate thermoregulation of body and also the cellular responsiveness towards hyperthermia have allelic variants in the mammalian gene pool. Thus, both natural and artificial genetic selection can influence how heat stress affects reproductive function [18]. The coat colour, hair length-controlling genes, and heat shock tolerance in cells are all traits that could be chosen. It may be possibilities to boost up thermal tolerance and also increase fertility in summer by genetically modifying or changing the biochemical properties of the embryo prior to transfer [18]. The recognition of genes plays a vital role in increasing resistivity of cells towards heat shock might led to the transfer of these into heat stress through the breeds sensitivity and transgenic techniques, resulting in cattle's with increasing resistance capacity to defect the heat stress. The selection of breeding animals would need to be given further thought. The performance-based livestock selection and the selection of best breeds based on the phenotypic behaviour with cost effective significant traits like high growth rates, has been practised for decades whereas, farmers can continue to selection of replacement breeds on the basis of individual results cost effective and based on their profits in significant traits in the coming years. According to [13], while genetic improvement initiatives continue to emphasise these economically significant traits, there is a risk that this could lead to a decrease in thermo-tolerance resistivity due to the connection between cattle productivity and also rising metabolism of heat output. The enhancement in metabolic heat output decline the thermo-neutral zone of the animals, which combination with seasonal variation, will make handling cattle in hot weather more difficult.

8.3 Nutritional management

To reduce heat generation through nutrient utilisation inside the animal, choose and feed new, palatable, and high-quality forages as much as possible, feed ingredients with a high digestibility [18]. The animals that are stressed need carbohydrates that can be fermented quickly. The essential ingredients should have buffering capacity such as sodium bicarbonate (NaHCO3), magnesium oxide (MgO) and sodium sesquicarbonate (Na3H (CO3)2) to maintain a natural atmosphere by effectively lower the occurrence of acidosis in the rumen, which is a frequent occurrence in hot weather [18] even if they are not eating as much feed as they need, early lactation cattle's effected to heat stress can go even deeper into increase in negative energy balance. As a result of altered follicle growth and decreased oestrus activity, they are more likely to have poor reproductive efficiency. Any of the symptoms of heat stress can be reduced by feeding high-quality forages and healthy rations. Since potassium is the primary component of sweat gland secretion in cattle, it should also be increased in their diet. As compared to fibre and carbohydrates. The intake of fats in diet is more beneficial because it help to reduction of heat and lowers the metabolic heat. In heat stress conditions, the dry matter easily digestible and also observed decrease in protein-energy ratio. In heat stressed cattle's, feeds on superior quality low-degradable protein has been observed to increase milk productivity as a result, both the amount and type of protein consumed by heat-stressed cattle's and buffaloes are critical. By using supplemental niacin to cattle's diet can also help them cope with heat stress. The Palm oil supplementation increased DMI while lowering heat stress signs [5]. The NEBAL was strengthened by feeding conjugated linoleic acids during heat stress, but milk fat was depleted at the same time. Lipoic acid has been shown to have antioxidant and energetic-metabolism-promoting properties [5]. The Exogenous antioxidant nutrient supplementation such a vitamin C, A, and E, as well as trace minerals including zinc (Zn), manganese (Mn), copper

(Cu), selenium (Se), chromium (Cr) and others, may be used to decline the adverse effect of environment [5]. The elements such as B-complex vitamins, ascorbic acid, tocopherol, rumen-protected by Niacin and Nicotinic acid [5] have all been found to be helpful. Thiazolidinedione's (TZDs) can boost HSP development [5] increase glucose utilisation [5] and boost energetic metabolism, making them a viable heat stress strategy. Dietary betaine, like TZDs, might be a better alternative in heat stressed lactating cattle's [5]. In heat stressed lactating cattle's, chromium supplementation has been shown to increase energy metabolism and performance [19]. There is evidence of the development of reactive oxygen species (ROS) by embryos growing at high body temperatures is one of the causes of embryonic death in heat stressed animals [13]. Efforts to increase the fertility of lactating cattle's subjected to heat stress by administering antioxidants have had mixed results [13].

8.4 Embryo transfer

The heat stress shows adverse impact on embryos greatest sensitivity, however, occurs during the early stages of embryonic development which leads to reduced pregnancy.. The embryos develop some tolerance capacity towards heat stress at time of embryonic development (morula or blastocyst stage). As a result, using embryo transfer of frozen embryos harvested from non-heat stressed cattle's, it might be potential to raise to pregnancy rates in heat stress effected cattle. There are multiple number of adverse effects of heat stress on the pregnancy of cattle (**Figure 3**). Many recent studies have shown that embryo transfer can be used to avoid embryonic death within the first seven days of development, when the embryos are more vulnerable towards heat stress. At the time of the summer, the technique has the potential to increase pregnancy rates dramatically. Furthermore, studies advances in improving embryo heat stress resistance through genotype modification and the accumulation of endurance factors like insulin growth factor-1, which help provide a protection to cells from following types of stresses, can boost pregnancy rates with embryo transfer even more. However, embryos are more responsible towards heat stress early pregnancy and also cooling the inadequate number of instance. At the time of the sensitivity of embryo at peak under heat stress shows enhancement of the pregnancy ratios moderately. As example of the rate of pregnancy towards artificial insemination for cattle's that were chilled for eight days after acceptance of prostaglandin F, (PGF2) was 16% as compared to 6% for control [20]. The limited cooling is not considerably useful for rate of increasing rate of pregnancy due to heat stress. The cooling was started on heat stressed follicles function and stopped at later at the time of pregnancy. The following conditions have hindered widespread trade implementation of embryo transfer for decline the rate of heat stress [21]:

1. The dairy heifers act as donors can origin their initial parturition to be overdue, resulting in lower productivity.

2. The embryo quality and ambient temperature have a negative relationship, the form up good to the superior qualities of embryos viabilities when the most needed.

3. The improvement of embryos is a time-consuming and costly procedure.

4. When it comes to frozen embryos, only developed in vivo have high percentage of pregnant cattle when compared to artificial insemination.

5. The in-vitro production of embryo cost is lesser than compared to in vivo embryos production. However, improved embryos are produced only with help of in-vitro technique. They are transferred fresh in cattle's.

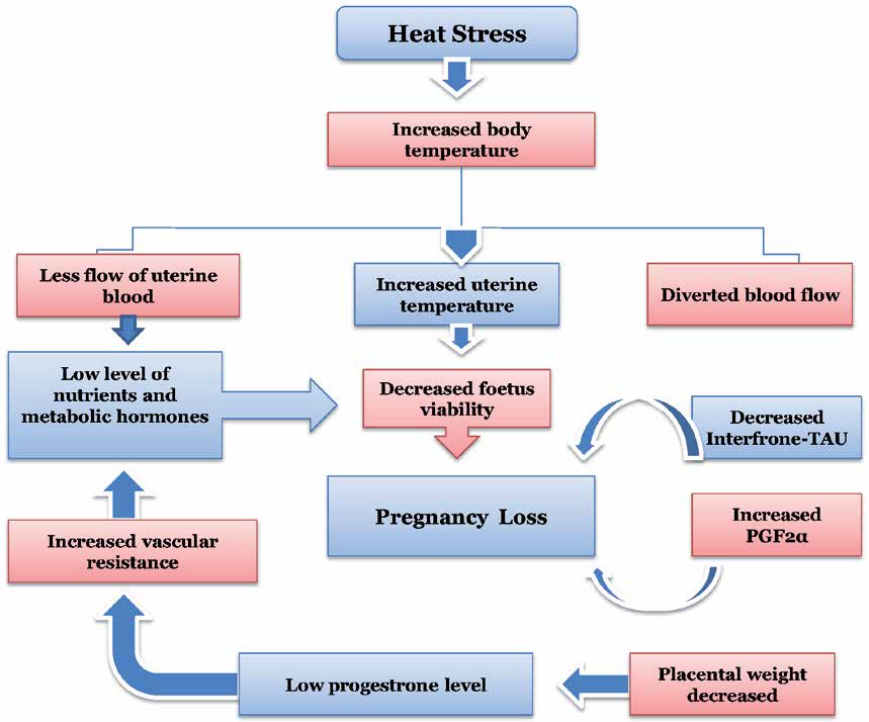

Figure 3.
Impact of heat stress on pregnancy of cattle.

8.5 Environmental strategies

In general, cattle's environmental strategies are a growing field in bovine science that is receiving further become aware of climate change. It aims to create appropriate micro-climate for most favourable production by minimising negative environmental effects on cattle's production organisation. The primary methods of changing the atmosphere can be divided into three categories [22]:

i. Creating a shady environment.

ii. Methods for evaporative cooling.

iii. Fogging systems use fine water droplets that quickly evaporate and disperse into the air stream, cooling the surrounding atmosphere.

8.6 Adaptation and acclimation

The all cattle's have capacities to get adapted in climatic surroundings which very essential to remember. Cattles may change their pattern of behaviour, their physiological, and order of morphological characteristics, in reaction to the temperature or climatic changes [12]. All species have endurance type of strategies to decline the heat stress over the entire body. Adaptation and acclimation are two coping strategies that animals have created. Although the terms adaptation and acclimation have completely dissimilarities in meanings, they can also be interchangeably [12].

8.6.1 Acclimation

The animals phenotypic reaction to an individual stressor in the environment is known as acclimation [23]. However, it is uncommon for only one environmental variable to adjust over time in natural environments. Acclimatisation is the adaptation of an animal to a variety of stressors in its natural environment [12] as a result, acclimation and acclimatisation are not termed as an evolutionary adaptation or natural selection, which are characterised as changes that allow for preferred selection of animal on basis of phenotype and also based on their genetic component that is conceded down to the next generation. If environmental stressors are eliminated, the altered phenotype of acclimated animals will return to normal. On the other hand, Animals are genetically adapted to their climate on the basis of their requirements. In other words, it's a homeostatic system triggered by the endocrine system that cause impact upon the cellular activities, metabolic activities and further alteration in systemic, which permits the animals show adaptability towards heat stress and also overcome to it [12].

At consequence, acclimation can be thought of as a mechanism that occurs over the course of a lifetime, in which constant exposure to a specific stressor, such as extreme hot weather, causes biological changes, improving the fitness of the individual animal to live in climatic conditions [12]. There are three functional distinctions between acclimatory and homeostatic or "reflex" responses, according to Collier and Zimbelman [23].

1. It takes a lot longer for the response to happen (months and years).

2. In the acclimation initial pathway from the central nervous system to the effector cell, acclamatory responses are usually linked to hormones.

3. The acclimatory effect alters an effector cells and organs capacity to respond to environmental change.

As previously mentionesd, these acclamatory responses are typical of homeorhesis processes, and the net result is to synchronise metabolism in order to achieve a recent physiological state. As a result, the metabolism of the seasonally adapted animal differ in the winter from the summer. The Heat stress adaptation is thus, a homeorhesis process involving alter the patterns of hormonal signals that manipulate target tissue sensitivity to environmental stimulus. The improved genetic quality selection of heat stress tolerant genotypes would result from a better understanding of this mechanism.

8.6.2 Adaptation

Adaptation is described as a biological change that occurs over generations as a result, of continuous stressor exposure and favours genetic assortment in an inhabitants to support species endurance [12]. For example Bos- indicus of tropical climates, earlier evolved in tropical climates with elevated temperature along with humidity as a result, they have a range of genetic variations that promote thermo-tolerance [12]. Thus, the ability of Bos-indicus breeds to survive in tropical environments on the basis of their requirements and adaptabilities towards it. They have evolved over generations. The climate conditions has the capability to oblige 'natural' selection for heat tolerant cattle in grazing breeding herds despite

of assortment forces obligatory on the inhabitants. The generations are developing succeeding capacities to adapt them in warmth environment. The progenies developing heat resistivity potential with climatic circumstances. However, it is difficult to reach to conclusion in the case of bovine because of long interval in the productivity. When cattle come in contact of acclimation and adaptation the adapt the degree of resilience. The acclimation and adaptation, when covmbined with the use of mitigation options, which has the ability to improve cattle performance and productivity during the high heat stress.

9. Conclusion

Heat stress as an effect of climate change, would inevitably is the reason heat stress in all farm animals, affecting their reproductive abilities. The impacts of heat stress on both females and males were explored in detailed in this chapter as well as male reproductive. This chapter also discussed mitigation measures that should be considered in order to avoid financial losses caused by environmental pressures on bovine reproductivity. Fortunately, there are managed methods for mitigating the severity of heat stress on bovine reproductivtiy. The involvement of cattle's in climate controlled environments, using the techniques of artificial insemination protocols that conquer the detection of poor oestrus, embryo transfer method and implementation of embryo to avoid damage in oocyte and earlier fertilisation. The heat stress can also causes the embryo to develop abnormally. To aid ruminants cope with harmful conditions, management options such as strategic technique are being use of providing the shades along with wind covering, attachment of sprinklers and providing ventilation at the time period of extreme heat stress should be considered. The strategy of diet intake, in addition to these steps, can be advantageous for ruminants facing environmental challenges also; there are possibilities for manipulating animal genetics to produce a more heat-resistant animal. The animals have genes for body temperature control and cellular tolerance to high temperatures. The temperature, as well as the recognition and genes assimilation in to breeds which are heat-receptive that does not decreases the reproduction, will be a great accomplishment. On the other hand all animals can adapt to their thermal surroundings through adaptation and acclimation. In response to temperature, animals can alter their patterns of behavioural, alter the physiological, and changes the pattern of morphological characteristics, or a combination of these towards heat stress.

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Chapter 9

An Assessment and Control of AFM_1 in Milk and Main Dairy Products in Lahore, Pakistan

Umaar Afzal Gill, Aneela Zameer Durrani and Muhammad Usman

Abstract

The main objective of this study is to investigate the presence of Aflatoxin M_1 (AFM_1) in local and processed milk and main dairy products available in Lahore. Total 60 milk samples and 120 samples of dairy products including butter (n = 30), cheese (n = 30), cream (n = 30), and yogurt (n = 30) were collected. Milk samples were collected from 3 different sources i.e. unprocessed milk from local milk shop (n = 20) and a local dairy farm (n = 20), and processed milk sample from a commercial shop (n = 20) while samples of each dairy product were also different i.e. processed (n = 15) and unprocessed (n = 15). Milk samples were analyzed using kit method while dairy product samples were analyzed by high performance liquid chromatography (HPLC) technique equipped with fluorescence detector (HPLC-FLD) followed by immunoaffinity column clean up. In second phase of the study, efficacy of three different toxin binders was compared and analyzed. The results showed that AFM_1 was detected in 16.7% of processed butter samples, 33.3% of processed cheese samples, 13.3% of local cream samples and 26.6% of processed yogurt samples and these samples exceeds European Union (EU) permissible limits of 0.05 ppb with mean concentration 0.090 ± 0.180 μg/kg and 0.000 ± 0.000 μg/kg for processed and local butter samples, 0.350 ± 0.606 μg/kg and 0.000 ± 0.000 μg/kg for processed and local cheese samples, 0.000 ± 0.000 μg/kg and 0.542 ± 1.085 μg/kg for processed and local cream samples and 0.552 ± 1.001 μg/kg and 0.000 ± 0.000 μg/kg for processed and local yogurt samples, respectively. Moreover, milk samples showed highest AFM_1 (62%) in local unprocessed dairy farm followed by samples from local milk shop (51%) and commercial dairy farm (31%). In addition, therapeutic efficacy of three different types of toxin binders showed that the toxin binder which had yeast wall (75%) and algae (25%) is the best to control AFM_1 under field conditions. Overall, results of this study are valuable for dairy farmers on one hand and law enforcement authorities on the other to comprehend and control AFM_1 problem in milk and main dairy products.

Keywords: AFM_1, Milk, Dairy products, HPLC, Toxin binders

1. Introduction

Milk and dairy products both are vital part of human nutrition and ideal sources of nutritional components because of their biochemical complexity for supplying

essential mixture of proteins, vitamins, calcium, amino acids and antioxidants [1]. Pakistan is 4th largest milk producing country in the world and produces 45 billion liters per year [2]. The extensive and vast dairy industry of Pakistan faces a lot of problems including Aflatoxin M_1 (AFM_1). AFM_1 are playing negative impacts on animal production as well as dangerous for human health [3, 4].

AFM_1 is a monohydoxylated product of Aflatoxin B_1 (AFB_1). When lactating mammals consume AFB_1 contaminated feed then production of AFM_1 becomes enhanced. After ingestion of AFB_1, hydroxylation reaction is occurred on tertiary carbon of difuran ring system which yields AFM_1 [5–7]. The biotransformation frequency of AFB_1 to AFM_1 in excreted milk is different in all lactating animals. But AFM_1 start producing in milk within 12 – 24 hours after AFB_1 ingestion from feed [8].

80% people especially children consume dairy products as an important part of their diet [9]. But it is a dejected reality that dairy products are compromised badly because of mycotoxins. These fungal toxins not only destroy these dairy products but also produce dreadful disease which causes chronic diseases in the consumer. The World Health Organization (WHO) has endorsed the depletion of Aflatoxins in food by establishing tolerable limits for Aflatoxins to fulfill Farm-to-Fork principle.

International Agency for Research on Cancer (IARC) which is specialized cancer agency of World Health Organization (WHO) has categorized aflatoxins B_1 and M_1 into group 1 carcinogens [10, 11]. Therefore, different international organizations and countries have established standards for AFM_1. The European Commission Regulation 1881/2006 set permissible limits for AFM_1 in milk and dairy products of 0.050 µg/kg [12, 13]. According to Codex Alimentarius Commission permissible level of AFM_1 in butter is 50 µg/kg and in cheese is 250 µg/kg [14]. So, permissible limits of AFM_1 vary in milk and dairy products in different countries. But many countries including Pakistan also have no proper safety and regulatory limits and levels for AFM_1 in milk and dairy products [15] which may be due to oversight of policy makers with negligible research on aflatoxins.

Hence, this study was designed to gauge AFM_1 problem in milk and dairy products in and around Lahore from different sources. In the second phase of the study, efficacy of three different toxin binders were compared in a local dairy farm. The outcomes of the study will help dairy farmers on one hand and law enforcement agencies on the other to understand the gravity of AFM_1 problem in milk and main dairy products, and formulate strategies to control it.

2. Material and methods

2.1 Sample collection

Sampling strategy is comprehensively discussed in **Table 1**. After collection, samples were transported in icebox to University of Veterinary and Animal Sciences, Lahore where these were stored at −4°C till further processing.

2.2 Sample processing

2.2.1 Processing of milk samples through rapid test kit

Rapid test kit detects AFM_1 to the limit of 0.5 ng/ml-ppb. The kit was used according to manufacturer instructions. Briefly, 200 µL milk samples were pipetted into reagent microwell and after mixing, these samples were incubated at room

Sample	Sample category
Milk (n = 60)	10 ml unprocessed milk from local milk shop (n = 20)
	10 ml unprocessed milk local dairy farm (n = 20)
	10 ml processed milk sample from a commercial shop (n = 20)
Butter (n = 30)	50 g Processed butter samples (n = 15)
	50 g unprocessed butter samples (n = 15)
Cream (n = 30)	50 g processed cream samples (n = 15)
	50 g unprocessed cream samples (n = 15)
Cheese (n = 30)	50 g processed cheese samples (n = 15)
	50 g unprocessed cheese samples (n = 15)
Yogurt (n = 30)	50 g processed yogurt samples (n = 15)
	50 g unprocessed yogurt samples (n = 15)

Table 1.
Sampling strategy.

temperature for 2 minutes. Then dipstick was inserted in each sample and incubated for another 5 minutes at the same temperature. After that dipstick was taken out from each sample and samples were interpreted. There would be two lines i.e. test line (T) and control line (C) on dipstick. If T > = C then sample was considered negative while if T > C or there was no test line then it was considered as positive.

2.2.2 Processing of samples of dairy products through HPLC

a. Chemicals and Reagents:

AFM_1 standards (10 μg/l in acetonitrile), Celite and HPLC grade acetonitrile of Sigma–Aldrich, Steinheim, Germany and Immunoaffinity column Afla^{Tm} of VICAM, USA were used.

AFM_1 standard curve or linearity curve was prepared by diluting the standards with acetonitrile at 0.05, 0.1, 0.2, 0.3, 0.4 and 0.5 μg/ml concentrations and stored in caped vials in refrigerator at – 4 C.

b. Samples Extraction:

5 g sample of each products and 5 g Celite mixed with 40 ml of dichloromethane in a 50 ml falcon tube. Then centrifuged at 21,000 rpm for 5 mins. After centrifugation the supernatant was separated and evaporated in water bath at 80 C. After evaporation the beaker was shifted in ultrasonic clean-up for 5 min. Then residues in beaker were dissolved in 10 ml mixture of methanol, water and n-hexane with the ratio of 3:5:2. Then 15 ml of this solution mixed by vortex mixture and again centrifuged at 21,000 rpm for 5 mins. After this, aqueous filtrate was passed through immunoaffinity column. The column was washed with 10 ml of water to remove toxins. After this column again washed with 2.5 ml of acetonitrile to get the final extract. This extract then dry under nitrogen steam at 40 C. After evaporation residues were dissolved with 2 ml mobile phase and mix by using vortex mixture. Finally, 20 μ/l sample was injected in HPLC for the analysis.

Groups	Type of toxin binders	Dose rate
A (n = 10)	Clay based toxin binder	100 g/40 Kg feed
B (n = 10)	Whole yeast-based toxin binder	1 g/40 Kg feed
C (n = 10)	Yeast (75%) + Algae (25%)	10 g/40 Kg feed

Table 2.
Therapeutic trials in different groups.

c. HPLC Conditions:

The HPLC used for the analysis was a Shimadzu LC-10A series (Japan) with the fluorescence detector (HPLC-FLD) having excitation wavelength of 365 nm and emission wavelength of 435 nm.

2.3 Therapeutic trial

Three different toxin binders were used in respective groups A, B, and C each having 10 AFM_1 positive animals. The **Table 2** showed types of toxin binder and their dose rates. Each toxin binder was used on the daily basis for 7 days.

2.4 Sample collection and processing after therapeutics

At days 2nd, 3rd, 4th, and 7th 10 ml milk samples from each animal were collected in plain vacutainers and serum was extracted through centrifugation. After that milk samples were checked by using AFM_1 rapid test kit.

2.5 Statistical analysis

Collected data will be statistically scrutinized by SPSS 20.0 software and t-test as well as Chi square test was used to analyze the results.

3. Results and discussion

The results of this study were comprehensively described in **Tables 3** and **4**. **Figure 1** showed the linearity curve of AFM_1 standard concentrations of 0.05, 0.1, 0.2, 0.3, 0.4 and 0.5 μg/ml. The method showed linear response $R^2 = 1$.

Table 3 shows the level of AFM_1 of all dairy products that exceeds the tolerable limits (0.050 μg/kg) established by European Commission Regulation.

Our results concluded that 33.3% of processed butter sample showed positive recovery of AFM_1 with range concentration above EU limits (0.050 μg/kg) while no local butter sample with AFM_1 toxicity was found (**Table 3**). These results are only in agreement with a study conducted by Fallah et al. [16] who analyzed 31 butter samples and got 25.8% AFM_1-positive ones with range above permissible limits established by EU.

The similar trend was found in case of processed cheese samples which were found contaminated (33.3%) with AFM_1 (**Table 3**) whereas in another study the AFM_1 concentration was found much higher i.e., 78% [15]. There are many other such studies having positive percentages of AFM_1 higher than our results [16–18].

Similarly, analysis of processed and unprocessed yogurt samples showed that 26.6% of former were contaminated with AFM_1 above EU permissible limit whereas no sample in latter was found affected with these aflatoxins. Our results showed low positive percentage than documented by Iqbal and Asi [15] who found

Type of Sample	Group	Mean ± S.D	Range of Conc. of Aflatoxin M1 in ppb	Samples Exceeding EC limits (0.05 ppb)
Butter	Processed	.0900 ± .18000	.36	(5/30) 16.7%
	Local	.0000 ± .00000	.00	
	Total	.0450 ± .12728	.36	
Cheese	Processed	.3500 ± .60622	1.05	(10/30) 33.3%
	Local	.0000 ± .00000	.00	
	Total	.1750 ± .42866	1.05	
Cream	Processed	.0000 ± .00000	.00	(4/30) 13.3%
	Local	.5425 ± 1.08500	2.17	
	Total	.2713 ± .76721	2.17	
Yogurt	Processed	.5525 ± 1.00118	2.05	(8/30) 26.6%
	Local	.0000 ± .00000	.00	
	Total	.2763 ± .71889	2.05	

Table 3.
HPLC results of aflatoxin M_1 in dairy products.

Time of sampling			**After 2 days**	
Groups	Positive	Negative	Percentage	p-value ($p < 0.05$)
A	10	0	100%	0.000
B	9	1	90%	
C	3	7	30%	
Time of sampling			**After 3 days**	
Groups	Positive	Negative	Percentage	p-value ($p < 0.05$)
A	9	1	90%	0.000
B	7	3	70%	
C	0	10	00%	
Time of sampling			**After 4 days**	
Groups	Positive	Negative	Percentage	p-value ($p < 0.05$)
A	9	1	90%	0.000
B	6	4	60%	
C	0	10	00%	
Time of sampling			**After 7 days**	
Groups	Positive	Negative	Percentage	p-value ($p < 0.05$)
A	8	2	80%	0.000
B	6	40	60%	
C	0	100	00%	

Table 4.
Efficacy of different toxin binders in groups A, B, and C.

59 AFM_1-positive samples. Many other such studies also showed higher incidents of AFM_1 in yogurt samples than our study [19–21]. Lastly, results of unprocessed cream samples showed the highest concentration of AFM_1 whereas no aflatoxin

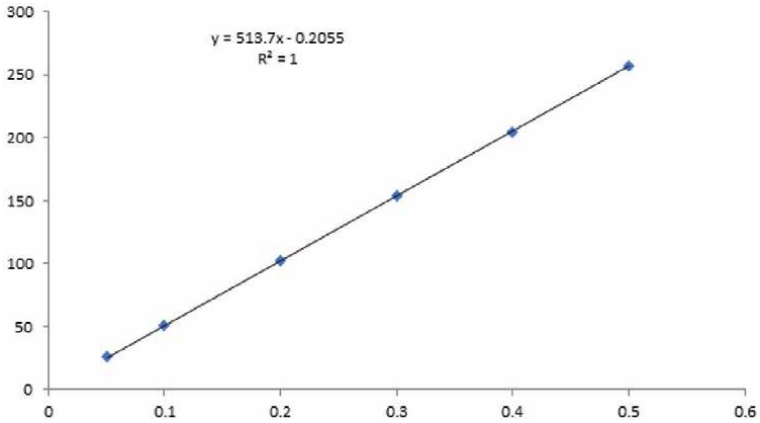

Figure 1.
Linearity curve of aflatoxin M_1 standard concentrations.

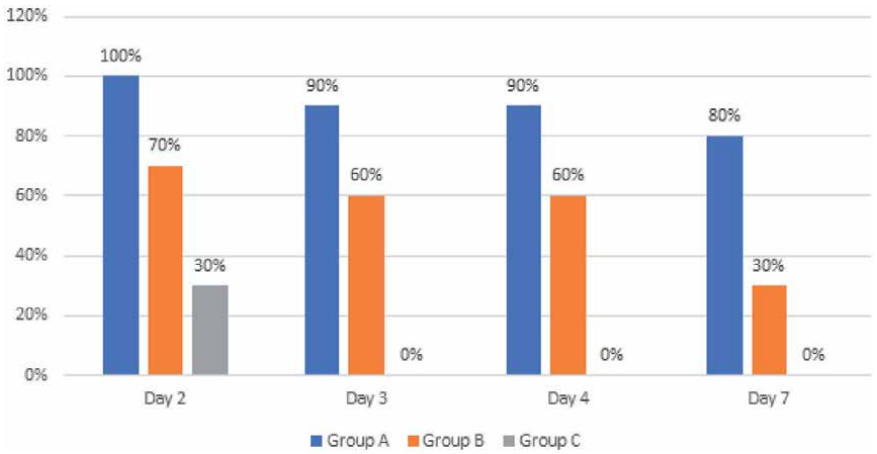

Figure 2.
Positive percentage in each group after offering toxin binders.

was detected in processed cream samples as shown in **Table 3**. These results have not only local impacts but high impact at global level too. Because at global level, particularly in underdeveloped and developing countries, the topic of aflatoxins in dairy sector impacting humans as well as animals is a neglected topic.

During the second phase of the study, milk samples collected on 2nd, 3rd, 4th, and 7th day showed significantly different efficacies of three toxin binders in groups A, B, and C. It was found that toxin binder used in group C had significantly higher (<0.05) efficacy as compared to those used in groups A and B. This toxin binder C had yeast wall (75%) in combination with algae (25%) so it showed best results and eradicated AFM_1 from all the animals after 48 h. The reason behind is the main role of yeast wall in the whole yeast which binds with mycotoxin and its binding ability is catalyzed by algae so the product having this combination provided the best results. Whereas, the clay-based toxin binder used in group A showed comparatively worst results in controlling AFM_1 due to their lack of binding with these mycotoxins as described by Chestnut et al. [22]. Other disadvantages associated with clay-based toxin binders are their probable interaction with the essential nutrients [23] and high inclusion rates [24]. On the other hand, whole yeast-based toxin binder showed significantly lower efficacy as compared to group C and higher

efficacy as compared to group A. The reason behind would be that whole yeast alone does not have good binding ability with mycotoxins so it also failed to control AFM_1, comparatively. The whole findings are summarized in **Figure 2**.

4. Conclusion and recommendations

The results of this study confirmed that processed and unprocessed milk and main dairy products i.e. butter, cheese, cream, and yogurt has significantly ($p < 0.05$) higher contamination of AFM_1 than EU permissible limit. Moreover, toxin binder having yeast wall (75%) and algae (25%) showed significantly ($p < 0.05$) higher efficacy to control AFM_1 in milk samples. The study depicts alarming situation of AFM_1 problem and stresses the need of establishing permissible limits of AFM_1 by the regulatory bodies to avoid their adverse effects on public health in Pakistan as well as in other developing countries. It is noted with grave concern that the issue of aflatoxins is one of the most discussed topic at global level but it neglected in underdeveloped countries. So, this study emphasizes the need of research on the impact and control of aflatoxins in milk, and other dairy products which are consumed by human beings.

Declaration

The authors declare no conflict of interest.

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Chapter 10

Device Diagnosing Health of Bovine

Sumi Kankana Dewan

Abstract

The research problem taken into consideration for study dealt with the design of a low cost hand-held ZnO based sensing device for testing blood serum of bovine (cow), to diagnose their health of liver and kidney by detecting four biological parameters in-situ. Zinc oxide nanoparticles were synthesised by chemical bath deposition method. Using transmission electron microscopy (TEM) and X-ray diffraction (XRD), the size of ZnO nanoparticles were determined. It shows a hexagonal wurtzite structure with an orientation along the direction (101). TEM images show various morphological changes of nanostructured ZnO. The average crystallite sizes of ZnO molecule is found to be 0.004 nm from XRD. The constituents of nano sized ZnO are found to be of Zn (57.27%), Cl (33.01%), C (8.04%) and O (1.68%) as obtained from EDS. The samples of blood serum of bovine, avian and caprine are characterised by transmission electron microscopy (TEM) and Benesphera Avantor Performance (Biochemistry Analyser). ZnO based sensing device is designed with the help of Arduino and Microsoft visual basic 6.0 version software. The resistance of blood serum is taken into consideration for carrying out the experiment. It has been measured after adding ZnO (1 μl) to blood serum of (1 ml) to detect four biological parameters – Serum glutamate pyruvate transaminase (SGPT), Serum glutamic-oxaloacetic transaminase (SGOT), Blood urea nitrogen (BUN) and creatinine of bovine more precisely. The device can indicate whether the blood serum of bovine have normal/diseased parameters. This device will also help the veterinarians in the field.

Keywords: SGPT, SGOT, BUN, creatinine, bovine, ZnO

1. Introduction

Many recent studies demonstrate that most nanoparticles (NPs) show an adverse or toxic effect on blood cells. Researchers have found that administration of ZnO nanoparticles (pH< 7) to whole blood samples and blood serums of animal and bird cause damage to the blood cells and tissues [1]. But NaOH are added to the acidic solution of ZnO to make the solution alkaline (pH=7.2) [2]. This alkaline nanostructured ZnO solution when added to whole blood and blood serum does not deteriorate the blood cells due to which biological parameters such as – Serum glutamic pyruvic transaminase (SGPT), Serum glutamic oxaloacetic transaminase (SGOT), Blood Urea Nitrogen(BUN) and creatinine can be more efficiently observed for different animals such as –cow (Class-Bovine).

Now a days nanoparticles based biosensors are introduced for obtaining the desired results more efficiently [3]. There are different types of biosensors which are incorporated with nanoparticles for diagnosing different problems in biological field [4].

2. General concept of biological parameters of animals

On Earth, there is no such living creatures which are free from diseases whether in case of human being or in case of animals, birds, insects or any other living organisms. It is true that cause of disease might be different. The reasons might be due to bad environmental conditons, due to lack of proper diets, due to hormonal imbalance, due to malfunctioning of organs, or it may be congenital or due to many more different conditions. Out of all diseases some are curable while few are beyond the control of doctors. Researchers are still doing researches to find out the solution of the remaining unsolved problems i.e. trying their best to make all the diseases curable. Some of the diseases can be controlled by the patient themselves by maintaining the biological parameters within the range which is safe for living a healthy life. But this will be possible only for human beings, not for the animals, birds and other living organisms. And it is the human beings who can also save the animals by taking care of the animals. Biological parameters such as creatinine, Serum glutamic pyruvic transaminase (SGPT), Serum glutamic oxaloacetic transaminase (SGOT) and Blood Urea Nitrogen are the parameters which gives an indication about proper functioning of liver and kidney.

Creatinine gives an accurate estimation for keeping a track on proper working of filtration processes of kidney. Formation of creatinine is shown in **Figure 1**:

Aspartate aminotransferase (AST) or SGOT and alanine aminotransferase (ALT) or SGPT are the enzymes that are present not only in the liver cells in large number but also in the muscle cells to a smaller number. If the liver gets injured or damaged, these enzymes are spilled into the blood by the liver cells, thereby raising the SGPT and SGOT enzyme blood levels and hence, indicating liver disease.

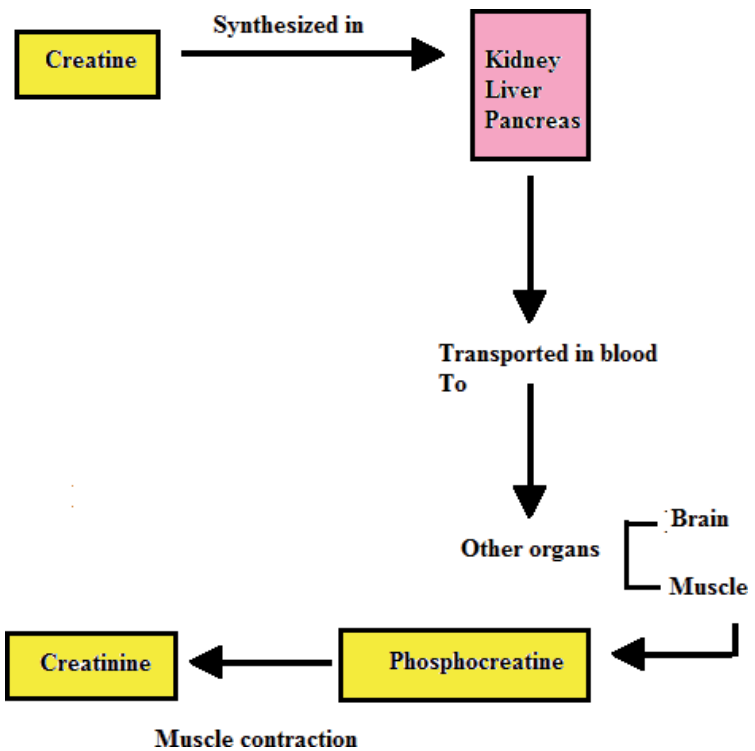


Figure 1.
Formation of creatinine.

BUN and creatinine levels gives a very accurate estimation of proper functioning of the kidneys. BUN measures urea level in the blood. To maintain a normal level of urea in the blood, both the liver and kidneys must function properly.

3. Motivation and layout of the research work

In India, cow, goat and poultry are tamed by human beings for fulfilling different needs. For milk production from cow, meat production from both goat and poultry and egg production from poultry. It should be our first priority to save our tamed animals from different diseases. After being consulted with Veterinary doctor, Dr. Jitendra Nath Dewan, Ex-Deputy Director, North Eastern Disease Diagnostic Laboratory, Khanapara, Guwahati, Assam, it has been found that once these tamed animals are affected with diseases affecting liver and kidney, then it becomes very tough to save them. So, focussed has been on those parameters whose values will give us the exact information about the proper functioning of liver and kidney.

In this chapter how the four different biological parameters of blood serum of bovine, i.e. SGPT, SGOT, BUN and creatinine are measured using nanoparticle ZnO based biosensing device has been discussed. This sensing device will be designed using ARDUINO board which will measure the resistance values of the serum samples of animal and bird. Corresponding to this resistance values, a look up table of above mentioned four biological parameters for the same serum samples will be maintained using Clinical Chemistry Analyzer. The microcontroller ATMEGA328P will be used to control the device in such a way that whenever this device will measure the average resistance value of any random sample of blood serum of bovine then it will immediately point to the nearest value of all the four parameters of the corresponding category of animal which will be stored in the look up table. Finally the result will be displayed on the device in–situ within seconds showing whether the health of the animal is in NORMAL /NOT NORMAL condition.

4. Sample preparation

All chemicals were purchased from the Emsure, are of analytical purity and used without further purification. All experiments were carried out in atmospheric pressure.

4.1 Synthesis of ZnO nanoparticles

Zinc Chloride is dissolved in 200ml deionised water in beaker of 250ml and placed on the magnetic stirrer and heated up to 70°C and at a speed rate of 450rpm as shown in **Figure 2**. Similarly, Polyvinyl alcohol is dissolved in 200ml deionised water in beaker of 250ml and placed on the magnetic stirrer and heated up to 70°C respectively. After this 180ml of Zinc Chloride solution is mixed with 180ml of Sodium Hydroxide solution [5] and to this mixture about 10ml of PVA solution is added in 500ml beaker, which is then placed on the magnetic stirrer and is heated upto 70°C for one hour. This solution is then kept inside a black box for one whole night for proper mixing and for cooling. After this solution is filtered using filter paper [6]. This will give a solution and powder of ZnO nanoparticles as shown in (**Figure 3**).

$$\mathrm{ZnCl_2} + Na(OH)_2 \rightarrow ZnO + \mathrm{NaCl_2} + \mathrm{H_2O} \quad (1)$$

Figure 2.
Zinc chloride solution is heated on a magnetic stirrer at speed 450 rpm upto 70°C.

Figure 3.
Beaker containing ZnO solution.

4.2 Preparation of slides

Slides were divided equally so as to make it fit for the size required for XRD test. After that slides are washed properly with distilled water and dried. Those slides were dipped into a beaker containing the solution of concentrated Nitric acid (conc. HNO_3) at an angle of 45°. After few hours slides were taken out and were again dried and are also covered by paper so as to avoid the accumulation of dust particles on the slides. Using dropper, few drops of the solution of ZnO is then dropped over

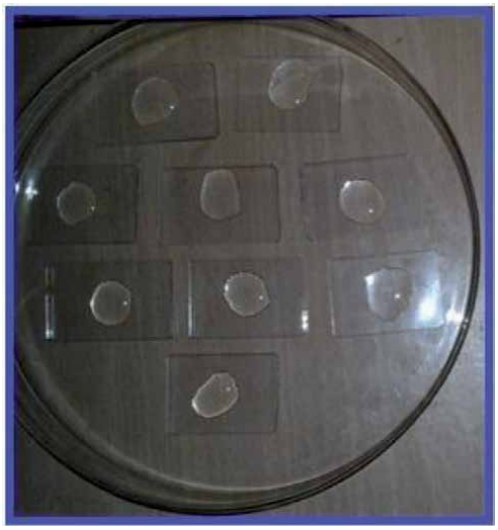


Figure 4.
Preparation of slides.

all the slides at the center properly in such a way that solution remains stagnant as shown in the **Figure 4**. Again this slides are kept covered for few days for drying.

4.3 Mixing of blood of animals and birds with ZnO solution

From recent studies it is found that most nanoparticles (NPs) show an adverse or toxic effect on blood cells. Studies show that administration of ZnO nanoparticles to whole blood samples and blood serums of animal and bird cause damage to the blood cells and tissues [7–9]. Researchers have investigated that microscopic ultrastructural changes occur in mice when ZnO nanoparticles is incorporated in its body [10].

Zinc oxide solution so prepared is best for use upto one month from the date of preparation but can be used for more number of days if it is kept in colder

Figure 5.
Left side: Blood sample of cattle: right side: ZnO mixed with blood sample.

Figure 6.
Left side: Blood serum of cattle: right side: ZnO mixed with blood serum.

temperature. As the solution so prepared is highly acidic (pH = 4.6), so after mixing it with sample of whole blood and serum respectively, it has been observed that colour of the blood and serum changes immediately, it changes from red to pink and colour of serum becomes milky which indicates that haemogolobin of that blood sample decreases as shown in **Figures 5** and **6**.

As pH of blood is 7.2, sodium hydroxide solution is again added drop by drop using micropipette of range 2-20 microlitre to the Zinc oxide solution to make pH value of that solution equal to or greater than the pH value of blood [11]. pH is recorded after every dropwise addition of sodium hydroxide solution to zinc chloride solution which is found as 7.5. After this the solution having pH greater than 7.5 is added to 1ml of blood of cattle. 0.5ml of Zinc oxide solution is added to the same proportion of blood kept for one night. No haemolysis is observed immediately. Again 0.5ml of Zinc oxide solution is added to the same proportion of blood serum of the same cattle and is kept for one night. No haemolysis is observed. But in the next morning again haemolysis is observed in both blood and blood serum.

Due to the occurance of haemolysis, pH of ZnO solution has been made exactly equal to the pH value of blood sample (i.e.7.2). 20µl of NaOH solution is added to 70µl prepared ZnO solution dropwise and after addition it has been observed that pH of ZnO solution becomes 7.2. Again 20µl of the ZnO solution (pH=7.2) is added to1 ml of blood of cattle as well to blood serum and immediately after addition as no haemolysis is observed as shown in **Figures 7** and **8** respectively, so, that sample has been used for manual test of blood to observe the parameters whether any changes have occurred.

And according to the blood test report of bovine given by the Central Instruments Laboratory, College of Veterinary Science, Khanapara, it has been found that RBC(red blood cells) counts shrinks and WBC(white blood cells) are vanished.

Again a new set of experiment is performed with the same ZnO solution. Initially only four parameters of Bovine has been tested. After that, ZnO solution (pH=7.2) of 2µl is added to the 1.5ml of the same blood serum of bovine and is

Figure 7.
Left side: Blood sample of cattle: Right side: 20μl ZnO mixed with blood sample.

Figure 8.
Left side: Blood serum of cattle: Right side: 20μl ZnO mixed with blood serum.

Figure 9.
Clinical chemistry Analyser (Benesphera).

tested using Clinical Chemistry Analyzer (Model C-61, Make Benesphera) in the Clinical Laboratory, Khanapara as shown in the **Figure 9**.

4.4 Measurement of resistance of blood serum of bovine

In the next set of experiment 1μl ZnO is mixed with 1ml of blood serum of 5 different blood serums of bovine collected from different specimen. The five different samples of blood serum of the three different species were then undergone clinical test for testing four biological parameters - Bun, creatinine, SGPT and SGOT. Also the above mentioned five samples of each three species were then mixed with ZnO and then again clinical test has been performed. It has been found that there is an increase in the values of the parameters after mixing all the 5 serums with ZnO. **Table 1** shows the comparison chart of biological parameters (after mixing with ZnO) of three species- bovine, avian and caprine respectively [12]. **Table 2** shows the normal range of biological parameters of bovine as obtained from clinical reference interval values given by Central Instrument Laboratory, College of Veterinary Science, Khanapara, Guwahati.

Samples	SGPT (U/L)		SGOT (U/L)		BUN (mg/dl)		CREATININE (mg/dl)	
	Serum	Serum + ZnO	Serum	Serum + ZnO	Serum	Serum + ZnO	Serum	Serum + ZnO
B1	46.2	51.3	37.4	40.2	64.3	73.6	1.8	2.1
B2	31.5	33.9	40.6	48.8	41.3	45.7	1.0	1.3
B3	32.3	36.8	54.6	59.2	67.3	71.4	2.1	2.5
B4	71.2	77.3	45.6	49.2	71.3	78.3	1.7	1.9
B5	17.4	21.3	26.3	33.5	44.6	48.2	1.1	1.4

Table 1.
Comparison of biological parameters of Bovine.

Normal range	Bovine
SGPT(U/L)	8–57
SGOT(U/L)	9–49
BUN(mg/dl)	18.8–55.4
Creatinine(mg/dl)	0.5–1.6

Table 2.
Normal range of biological parameters of Bovine.

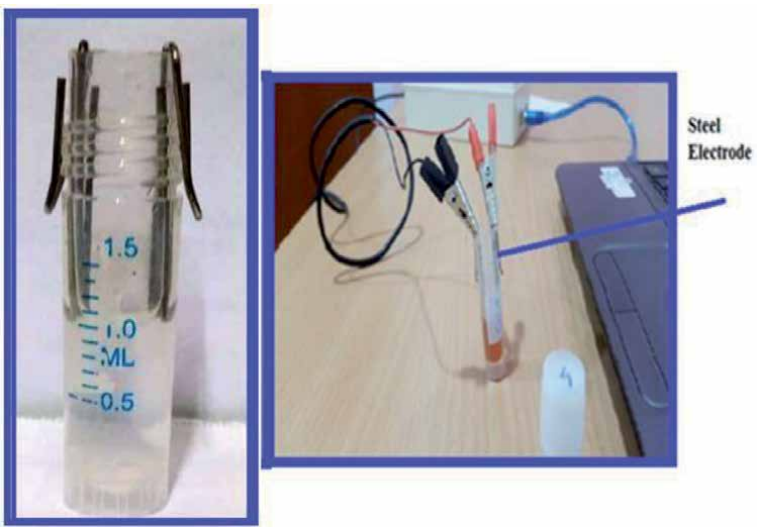


Figure 10.
Vial containing serum with steel electrode.

The resistance of 5 samples of blood serums and same 5 samples mixed with ZnO are measured using multimeter and also using Arduino as shown in **Figure 10** so as to check how resistance changes after mixing serum with ZnO. Arduino is an open source electronics board which is easy to use for programming. **Figure 11** shows the circuit diagram to measure the resistance of blood serum using multimeter and Arduino. **Tables 3** and **4** shows the values of resistances. It has been observed that resistance increases when serum is mixed with ZnO when measured in both the cases. i.e. using multimeter and using Arduino [13].

The experiment is carried out by inserting two electrodes of stainless steel at a distance of 0.81 cm into a vial consisting of serum. This electrodes are dipped into the vial of 1.5ml (as shown in **Figure 10**) in such a way that both the electrodes only touch the surface of serum. This particular set of electrodes are used for all type of samples after cleaning. The measurements of vial are taken using vernier calliper of zero least count are as follows:

- Outer diameter = 1.07 cm
- Inner diameter = 0.92 cm
- Inner diameter with clip (stainless steel) as electrode = 0.81 cm

The circuit shown in the **Figure 11(a)** has been used to observe the variation in the value of resistance of blood serum in the multimeter when current flows

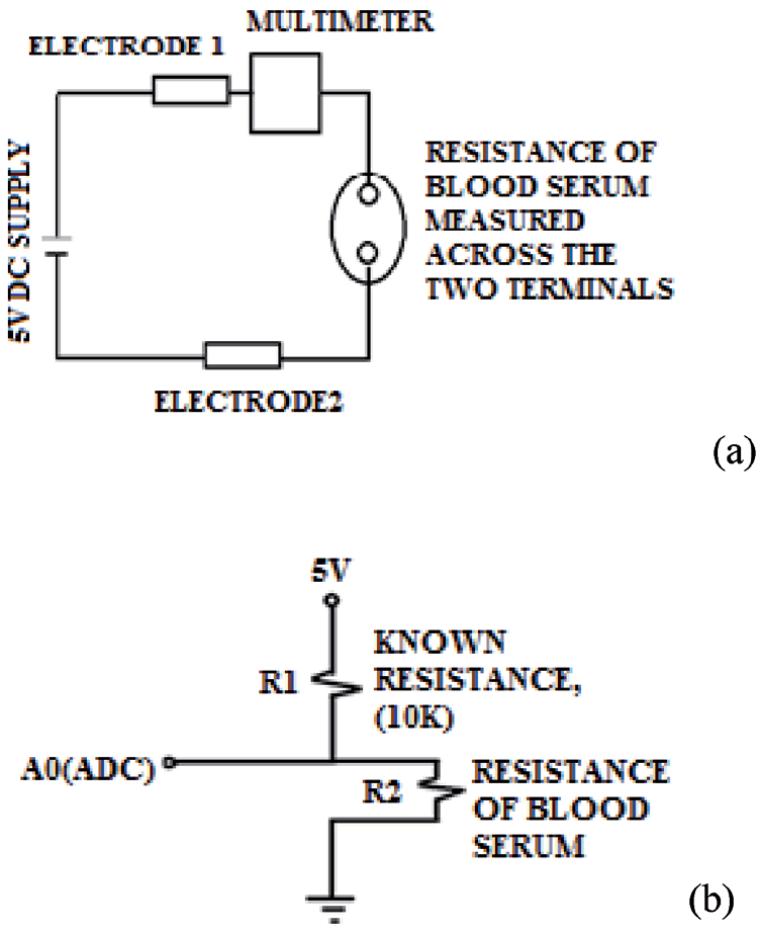


Figure 11.
(a):Circuit diagram of rmeasuring the resistance of blood serum using multimeter; (b): Circuit diagram for measuring the resistance of blood serum using Arduino.

Samples	Resistance (k)	
	Serum	Serum + ZnO
Bovine		
B1	7.24	10.35
B2	10.46	15.00
B3	12.07	13.44
B4	1.90	11.60
B5	17.00	18.00

Table 3.
Measurement of Resistance of 5 different samples of bovine using multimeter.

through the serum between the electrodes. These electrodes are connected to 5V dc supply so that when current flows through the serum there occurs a change in resistance of blood serum which is then further get stable at certain value. Again when nanoparticle ZnO solution is added to the same serum sample, then it has been observed that value of resistance measured by multimeter is greater than that

Samples	Resistance (Ω)	
	Serum	Serum + ZnO
BOVINE		
B1	27741.9	30497.76
B2	29106.5	30023.35
B3	25700.0	26204.20
B4	26204.2	27088.70
B5	28663.8	29106.50

Table 4.
Measurement of Resistance of 5 different samples of bovine using Arduino.

value of resistance measured without adding ZnO. We have used same set up for measurement of all the samples. Using circuit shown in **Figure 11(b)**, resistance of same samples are measured using Arduino [14]. Here, ADC gives voltage and current value and in this model we have taken into consideration the resistance mode, so using voltage divider rule the unknown value of resistance of blood serums has been found out as shown in the Eqs. (2) and (3):

$$V_{out} = V_{in} \times \frac{R_2}{R_1 + R_2} \tag{2}$$

$$R_1 = \frac{V_{in} - V_{out}}{V_{out}} \times R_2 \tag{3}$$

where, R_1 = resistance of blood serums, R_2 = known value of resistance i.e. 10 K, V_{in} = Supply voltage (5 V), V_{out} = Output Voltage (4335.9 mV).

It has been observed that resistance increases after addition of ZnO to 5 different samples of blood serum of three species [15].

4.5 TEM study of blood serum

The necessity to carry out this TEM study of blood serum sample (with and without adding ZnO is that the device which we were going to design is ZnO based biosensor in which we have to check the compatibility of ZnO with serum sample. Already it has been analysed that ZnO can be added to serum sample but the proportion must be maintained otherwise ZnO may damage the serum sample. It has been concluded that if 1 microlitre of ZnO is added to 1ml of serum sample then it does not alter the biological parameters of serum. In fact addition of ZnO will be more effective in designing a ZnO based biosensor as discussed in the previous section.

As per suggestion given by expert technician of NEHU Shillong performing TEM experiments, serum sample mixed with ZnO should be tested under biological sample category although ZnO comes under material sample category because we need to compare the TEM images of serum sample (with and without ZnO) basically to observe the existence of protein structure even after addition of ZnO so that we can proceed one more step to design our proposed ZnO based device of high efficiency.

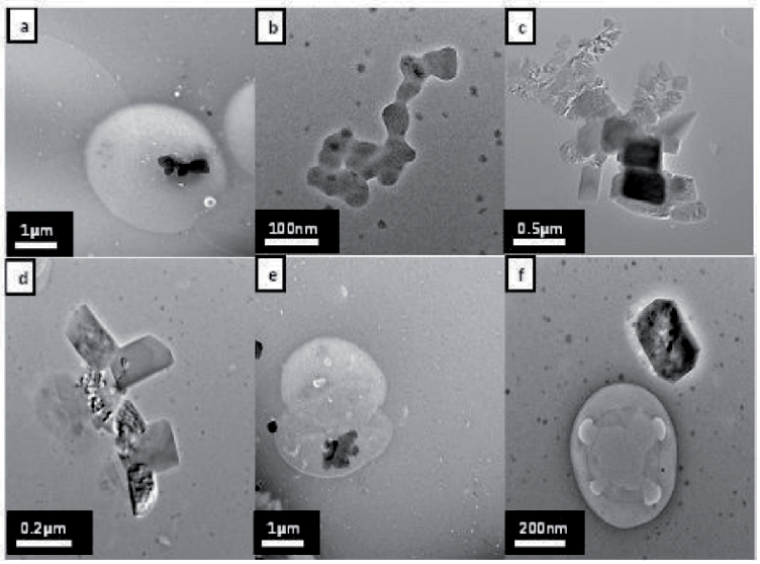


Figure 12.
TEM images of blood serum of bovine.

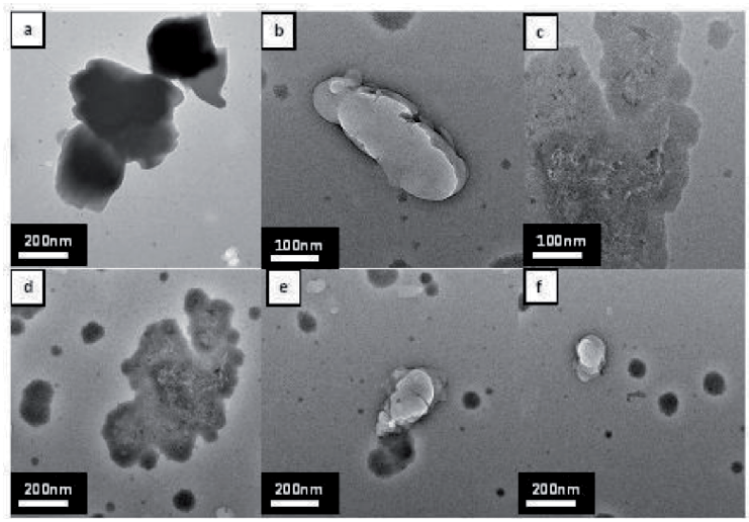


Figure 13.
TEM images of blood serum of bovine mixed with ZnO.

4.5.1 TEM study of blood serum of bovine (cattle)

To obtain TEM image of blood serum of bovine, 1ml serum sample is taken. The images showing protein structure, cell membrane are observed using TEM (JEOL-100CX) as shown in the **Figure 12**:

4.5.2 TEM study of blood serum of bovine (cattle) mixed with ZnO

TEM images are observed using TEM (JEOL-100CX) as shown in the **Figure 13** after mixing 1ml blood serum of bovine with 1 µl of ZnO using micropipette. The irregular black spots of serum proteins are observed [16].

In TEM study, the irregular black spots of about 30-40nm are serum proteins. The average size of serum albumin is 36 nm. Bubble like structure are the aggregated lipoproteins. It has been observed that mixing of 1μl ZnO with 1ml blood serum cause an increase in order of the biological parameters viz. BUN, creatinine, SGPT and SGOT and electrical parameter (resistance) which will help to analyse the variation of biological parameters of blood serum, after the incorporation of ZnO nanoparticle in it [17].

This study also tells us that the protein structure still exist in the images observed from TEM of blood serum of bovine mixed with ZnO same as that of the images observed from TEM of only blood serum of animal .

5. Experimental set-up

Blood serum is obtained from the blood which has been collected with the help of the veterinary doctor Dr. Jitendra Nath Dewan, Former Deputy Director, North Eastern Regional Disease Diagnostic Laboratory. Blood serum of cow, goat, poultry are mixed with nanostructured ZnO solution for measuring biological parameters SGPT, SGOT, BUN and creatinine. As it has been observed that there is an increase in the order of the biological parameters alongwith the increasing value of resistance of blood serum, when the blood serum is mixed with nanostructured solution. And based on this principle, circuit has been developed to design the sensor.

5.1 Circuit diagram

The circuit diagram has been shown below in the **Figure 14**. The basic components of the device are as follows:

a. Microcontroller–ATMEGA328P

b. LCD Module –20 x 4 display

c. I2CDriver

d. USB to TTL converter

e. PC/Laptop

f. Steel sensor probes

g. Resistance of known value-10 K

h. Five Push down tactile switches

i. Voltage regulator-7805

5.2 Operation of ZnO based sensor

The heart of the circuit is an 8-bit AVR series microcontroller ATMEGA328P. This particular microcontroller has been selected as it has an inbuilt ADC. The power supply can be provided, either by using 9-12V battery or by connecting through USB port, it can be driven or by using adapter. A very popular voltage

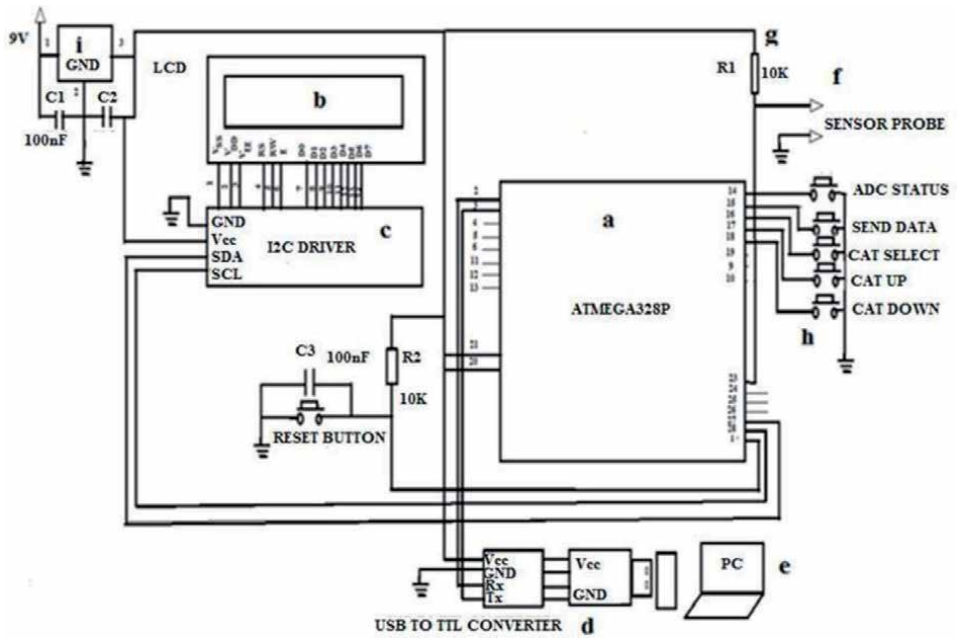


Figure 14.
Circuit diagram of ZnO-NP based biosensor.

regulator 7805 has been used to provide regulated +5V supply which gives regulated 5V with 1 Ampere current tracking. Two 100 nF ceramic capacitor are used across pin no.1 and 2 and across pin no.2 and 3 of 7805 to reduce harmonic noise or ripple.

A 20 x 4 LCD module has been used to display the calculated value and to display the result. Instead of using parallel communication, serial LCD (I2C protocol) has been used to simplify the circuit, which helps to display information from microcontroller to LCD.

Only two wires are enough for the communication, i.e., SDA (Serial Data) and SCL (Serial Clock) in case of I2C protocol. ATMEGA328P is also having inbuilt I2C support. Pin no.27 (PC4) of ATMEGA328P is SDA which is connected to the SDA pin of the LCD module for transfering the data as well as command from microcontroller to LCD serially. In I2C protocol, synchronous communication is implemented where both the device require a common clock source. In this circuit, the microcontroller is working as master and the I2C driver for LCD is working as slave. SCL pin is used for the clock pulse. Pin 28(PC5) of ATMEGA328P is connected to SCL pin of the I2C driver. Pin no.1 is assigned as "RESET" pin. For normal operation pin no.1 must be pulled up and then the device will restart if a low pulse is provided at pin no.1. Hence, a 10K resistance is connected from pin no 1 to Vcc.

In this device five push to on tactile switches are used for different purpose. To START or STOP the ADC reading, a switch is connected to pin no. 14 (PB0). To SEND the data from microcontroller to a computer system through a USB cable, as with is connected to pin no.15 (PB1).

The program executing in the computer is responsible for collecting the data from the microcontroller. This data are processed further and compared with the data already stored in database. The stored data are collected from the real sample. And data of 5 samples of bovine, avian and caprine are entered into the database. Whenever the computer receive a data from the microcontroller, it start searching for the nearest match in the sample table. To find out the nearest data, a simple mathematical formula has been derived. After retrieving the data, the application

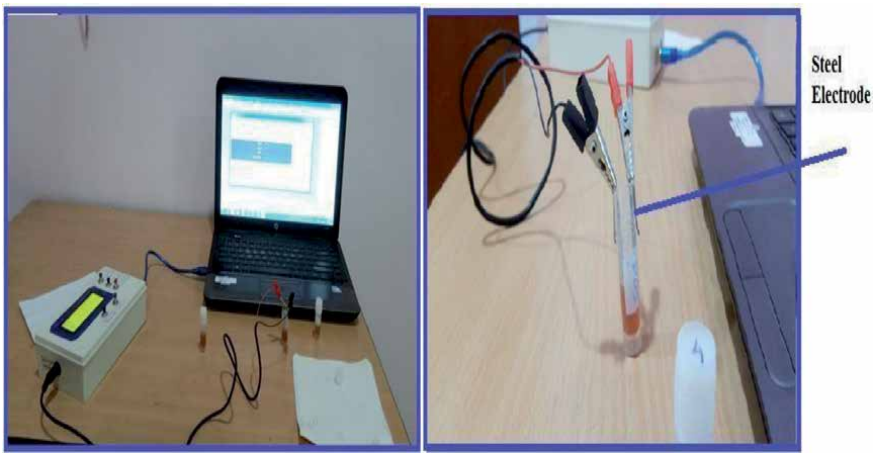


Figure 15.
Measurement using steel electrode.

program display the resultant value on the computer's screen and at the same time the data has been transferred to the microcontroller though serial communication.

The program executing in the microcontroller is responsible for retrieving the information from PC and to display the received data. USB to TTL converter has been used to transfer or communicate between PC and the hardware section. Steel probe has been used to get the resistive value of the sample .One probe is connected to ground and the other probe is connected to the ADC channel '0' of ATMEGA328P. To keep the ADC pin high at no load, 10K resistance has been used. Whenever the probe are placed on/in the sample, it returns different resistance value which is processed to predict the resultant.

Due to different resistive characteristics of different material, the probe material will affect on the result. Measurement can be taken by using electrode of any material (e.g. steel, lead) as shown in the **Figures 15** and **16** but the experiment is performed using steel electrode.

5.3 Arduino board

Arduino UNO board is the most popular board in the Arduino board family. In fact, it is the best board to start with electronics and coding. Some boards are

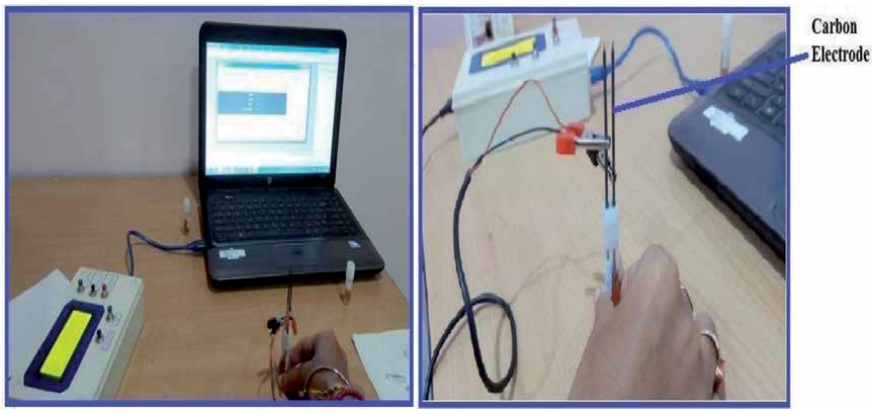


Figure 16.
Measurement using carbon electrode.

Figure 17.
Arduino board.

different from one another, but most Arduinos have almost same components in common as shown in **Figure 17**:

5.4 Datasheet ofATMEGA328

Figure 18 shows the datasheet of ATMEGA328P and also shows the input output mapped with Arduino UNO.

5.5 Microsoft visual basic

Visual Basic, is a basic programming language. Both simple and complex GUI applications can be created by programmers. **Figure 19** shows GUI created using Micrsoft visual basic 6.0 version and **Figure 20** shows an example.

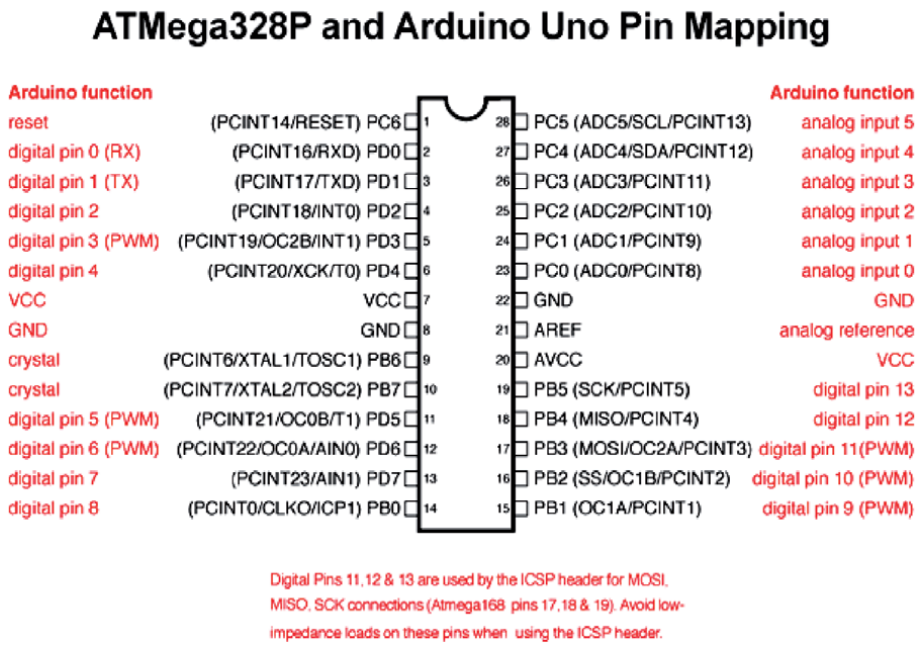


Figure 18.
Datasheet of ATMEGA328P.

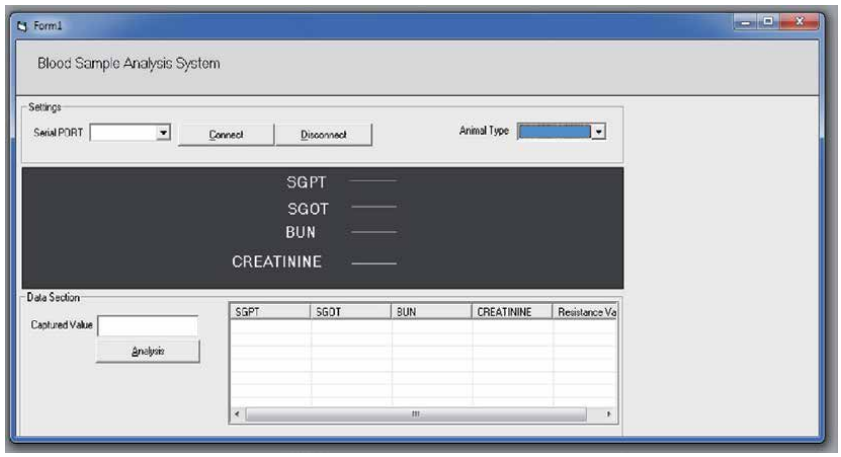


Figure 19.
GUI showing blood sample analysis system.

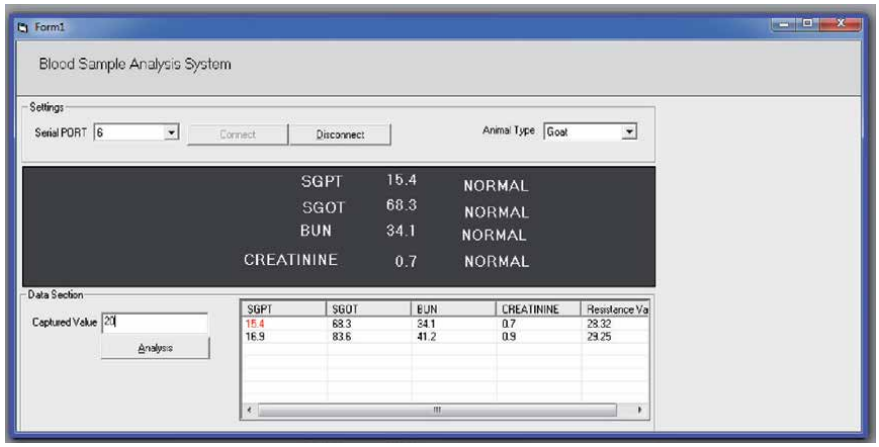


Figure 20.
GUI showing blood sample analysis system of caprine (goat).

5.6 Database management system

Database management system such as Microsoft access binds the Microsoft Jet Database Engine with a graphical user interface (GUI) and software-development tools.

Table 5 show the look up table of five different samples of blood serum of bovine, alongwith their normal ranges of all the four different biological parameters i.e. SGPT, SGOT, BUN and creatinine.

The resistance of five samples of bovine are measured respectively using the Arduino and their corresponding values of four biological parameters so measured by clinical test are maintained in Microsoft access as look up tables so that any serum sample of bovinewhen taken for test using the sensing device, it will read the analog value of resistance and will immediately compare that value of resistance with the look up table and if both the values of resistance get matched then corresponding four biological parameters will be displayed indicating whether the parameters are in normal range or not.

Cow	SGPT (U/L)	SGOT (U/L)	BUN (mg/dl)	Creatinine (mg/dl)	Resistance (K)
Normal range	8–57	9–49	18.8–55.4	0.5–1.6	—
1	51.3	40.2	73.6	2.1	30.5
2	33.9	48.8	45.7	1.3	30.02
3	36.8	59.2	71.4	2.5	26.20
4	77.3	49.2	78.3	1.9	27.08
5	21.3	33.5	48.2	1.4	29.10

Table 5.
Look up table of five different samples of cow (bovine).

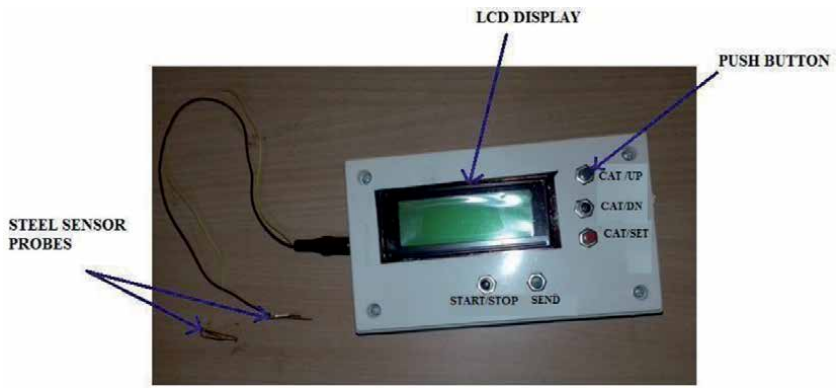


Figure 21.
Front view of ZnO based sensing device.

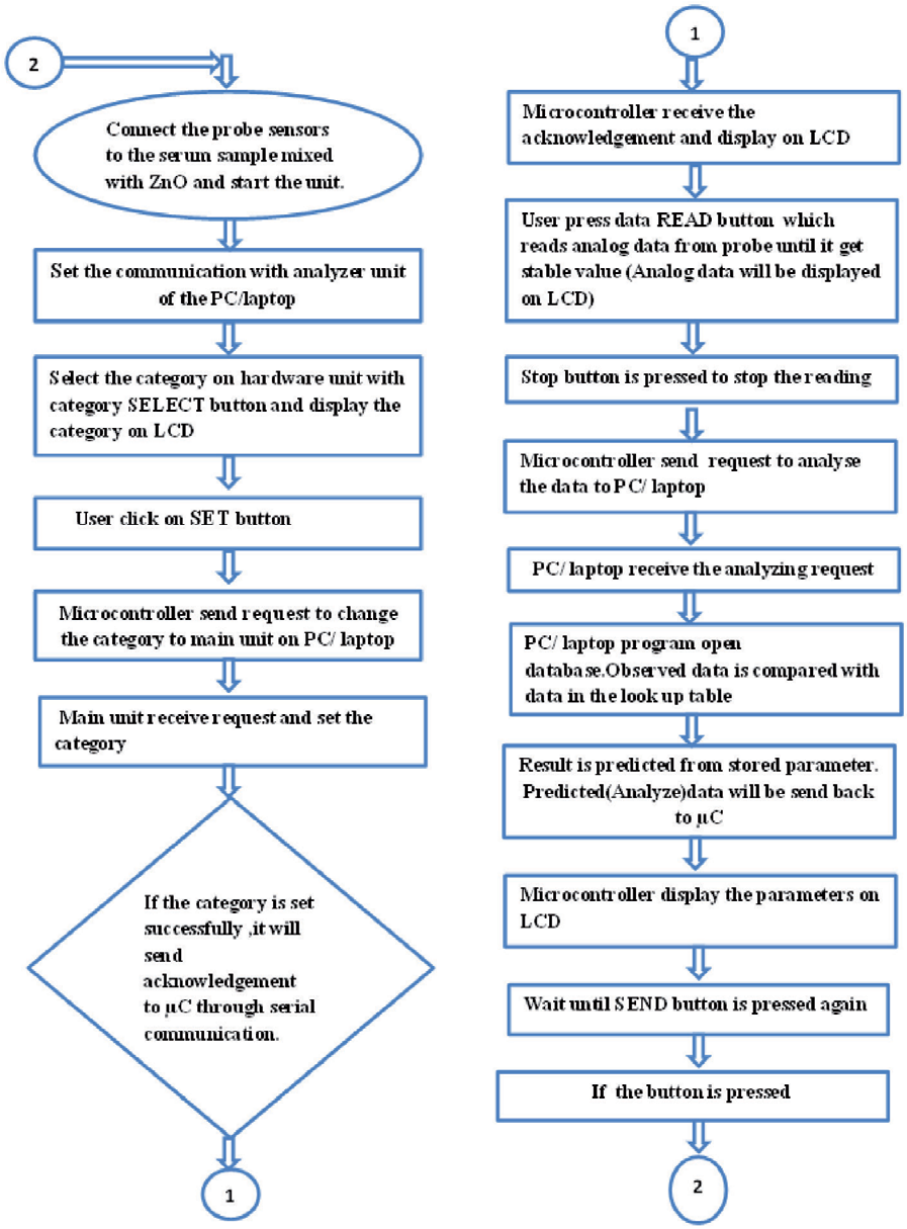
Figure 22.
Left hand side view of ZnO based sensor; right hand side view of ZnO based sensor.

5.7 ZnO based biosensor

ZnO based biosensor is designed as shown below. Front view, side view of the biosensor is shown in the **Figures 21** and **22**.

5.8 Flow chart showing the steps to take the readings of biological parameters

Flow chart below shows the steps to take the readings of the parameters of bovine, avian and caprine:



6. Measurement of biological parameters for bovine

Samples of bovine mixed with 1 µl of ZnO is tested with the device i.e. ZnO based sensing device which gives the reading of SGPT, SGOT, BUN and creatinine and also it tells whether it comes under the normal range or not. If the values does not lie within the normal range then owner of the patient should immediately consult a doctor. **Figures 23** and **24** show measurement of biological parameters of bovine.

6.1 Comparison chart

Samples which has been tested earlier in Clinical Laboratory one year ago are again used for clinical test (Clinic) and compared with the ZnO based

Figure 23.
Measurement of biological parameters of bovine.

Figure 24.
Biosensor displaying the biological parameters.

sensing device (Device). Serum samples belongs to the same bovine which has been collected in mass amount to carry out the test successfully to make conclusion.

6.1.1 Biological parameters of bovine (cow)

Samples of bovine (cow) are used for clinical test and compared with the ZnO based sensing device. Five blood serum sample are used which are denoted by B1. B2, B3, B4 and B5.

6.2 Statistical analysis and conclusion

Statistical analysis is done using IBM SPSS version 20 for the data of **Table 6**.

Standard deviation and standard error are evaluated using paired sample test as shown in **Table 7**. Also to check the efficiency of the device, correlation and t-test are carried out as shown in **Table 8**. Correlation test tells us about the similarity in the observations of both CLINIC values and DEVICE values and t-test tells us the difference in the paired samples i.e. CLINIC values and DEVICE values [13].

SAM PLES	SGPT(U/L)		SGOT (U/L)		BUN (mg/dl)		CREATININE (mg/dl)	
	Clinic	Device	Clinic	Device	Clinic	Device	Clinic	Device
B1	37.1	36.8	59.8	59.2	71.7	71.4	2.6	2.5
B2	77.8	77.3	50.4	49.2	78.6	78.3	1.9	1.9
B3	34.2	33.9	49.5	48.8	45.9	45.7	1.4	1.3
B4	36.9	36.8	59.5	59.2	71.6	71.4	2.5	2.5
B5	35.4	36.8	61.8	59.2	69.4	71.4	2.5	2.5

Table 6.
Comparison chart of biological parameters of bovine between clinical test and sensing device reading.

Paired Samples	Mean	No. of Samples	Std. Deviation	Std. Error Mean
BOVINE				
Pair 1 SGPT_Clinic SGPT_Device	44.28	5	7.59506	3.39663
	44.32	5	7.29872	3.26410
Pair 2 SGOT_Clinic SGOT_Device	56.18	5	5.76689	2.57903
	55.12	5	5.58856	2.49928
Pair 3 BUN_Clinic	67.44	5	12.52809	5.60273
BUN_Device	67.64	5	12.62351	5.64541
Pair 4 Creatinine_Clinic	2.18	5	0.51672	0.23108
Creatinine_Device	2.14	5	0.53666	0.24000

Table 7.
Paired sample test of bovine.

Paired Samples	No. of Samples	Correlation Test		t-test	
		Correlation	Sig	t	Sig
Bovine					
SGPT_Clinic & SGPT_Device	5	0.999	0	−0.116	0.913
SGOT_Clinic & SGOT_Device	5	0.987	0.002	2.566	0.062
BUN_Clinic & BUN_Device	5	0.997	0	−0.444	0.680
Creatinine_ Clinic & Creatinine _Device	5	0.995	0	1.633	0.178

Table 8.
Paired sample Correlation test and t-test of bovine.

From correlation test it has been observed that CLINIC values and DEVICE values are highly correlated as the significant values are less than 0.05 and from t-test it has been found that CLINIC and DEVICE values are almost similar as the significant values are greater than 0.05. Standard deviation and standard error mean value is also low which reveals that each sample is closer to the mean value. Correlation test and t-test helped us to conclude that the developed low cost handheld ZnO based sensing device is highly efficient for measuring the biological parameters- SGPT, SGOT, BUN and creatinine of bovine to diagnose the health of liver and kidney. This device is user friendly.

7. Future scope

A handheld low cost ZnO based biosensor can be developed using Rasberry Pi so as to make the device more efficient and portable. Rasberry Pi is a low cost small sized device that are good for software applications. Using Rasberry Pi, task of maintenance of database will be reduced as it has its own storage capacity. Hence, look up tables of bovine, avian and caprine of more than five samples can also stored in Rasberry Pi. Besides this I also intend to design a same design for human being so as to save peoples from being affected by liver and kidney related diseases.

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Section 4

Production

Chapter 11

Nutrition of the High-Yielding Dairy Cow

Petra Wolf

Abstract

In addition to genetics, health status and housing management, the milk yield of the dairy cow is also significantly determined by the feeding regime. In addition to the energy and nutrient supply, the dry matter (DM) intake plays a decisive role. This is influenced by many factors, such as the palatability of the feed, the energy density, the quality of the roughage (silage quality, microbiological status, contamination with mycotoxins) or the ration design in total. Water supply of cows is often forgotten, although it has a significant influence on the feed intake and thus the performance of the cows. This article deals with those factors, gives the latest recommendations and points out possible sources of error against the background of feeding not only according to the species-specific requirements but also according to their energy and nutrient needs, as required by the Animal Welfare Act.

Keywords: dairy cow, cattle nutrition, feed intake, feedstuff, nutritive related disorders

1. Introduction

In everyday life, the feeding of cattle is usually judged from an economic point of view: A feed fulfils its purpose if it supplies the animal in such a way that it achieves its performance and is also as cost-effective as possible. In the process, animal welfare is sometimes overlooked, although this in turn has an influence on performance, useful life and thus also on the economic viability of cattle farming.

Since the 1970s, the performance of dairy cows has increased by more than 35%. At the same time, however, the age of life decreased significantly [1]. Thus, about 30% of dairy cows already drop out in the first lactation. This is mainly caused by udder diseases (around 30% of mergers), but hoof and joint diseases as well as metabolic disorders (around 10% each) are also cited as causes [2]. There is no doubt that several factors are responsible for these developments and must be taken into account and optimised accordingly. However, the question arises as to the importance of feeding in this context. According to the Animal Welfare Act (§ 2), anyone who keeps, looks after or has to look after an animal must feed, care for and house the animal in a manner appropriate to its species and needs.

Here, the interlink between feeding and husbandry becomes clear. In order to ensure sufficient feed intake, the housing conditions must first be designed in such a way that the animal is able to carry out its physiological behaviour with regard to food and water intake as well as chewing behaviour. The design of housing follows the concept of the "five freedoms", which requires freedom from (1) hunger and thirst, (2) discomfort, (3) pain, injury and disease, (4) fear and stress, and (5)

the exercise of normal behaviour. The importance of this behavioural concept for the general performance of animals is underpinned by studies carried out within the AgroClustEr PHÄNOMICS at the University of Rostock and the Leibniz Institute for Farm Animal Biology. It was shown that different temperament types differ not only in their behaviour, but also in their metabolism [3]. Taking into account the species-specific (preferably temperament-specific) requirements helps to make optimal use of existing metabolic pathways.

In order to enable the animal to ingest sufficient quantities of feedstuff, there should be (except in the case of stations in which concentrated feed is offered) an animal to feeding place ratio of 1: 1 (control report 2015 for Lower Saxony and Bremen in accordance with Article 41 of EU Regulation No. 809/2014 for the on-the-spot controls on cross compliance [4, 5]). The feed offered must meet clear legal requirements. According to § 3 of the Animal Welfare Act, it is forbidden to offer feed that causes the animal considerable pain, suffering or damage. This certainly refers to contamination such as foreign bodies (stones, wire, etc.) or poisonous plants, but also to the hygiene status of a feed (e.g. yeast content, contamination with mycotoxins), which can have considerable consequences for the animal's well-being (e.g. tympania in younger cattle after ingestion of heavily contaminated silage). In addition, the ration design itself is also important, which will be discussed in the following chapters. Imbalances in the crude fibre and starch content of the feed, lack of synchronicity in the rumen, energy or mineral deficiencies as well as the use of less palatable feeds are examples to be mentioned here. Finally, the feeding technique is also important, which varies considerably in some cases and affects the quality of the cow's supply and thus their well-being.

2. Ration design: critical points and opportunities for failures

In the following, the general aspects of ration design for high-yielding cows and the problems that arise in practice will be discussed. Data from the service area, in which cases of damage caused by nutritive factors will be presented. In addition, results from ongoing scientific studies will be included accordingly.

2.1 Selection of suitable feed

Feed rations for dairy cows consist of roughage (basic feed) as well as concentrates and mineral feeds. The quality of the roughage already has a significant influence on the amount of feed consumed [6, 7]. When checking the hygiene status of grass silage (n = 109), 41% of the samples showed an increased yeast contamination, which is associated with poorer acceptance of the feed [8] and thus lower feed intake quantities (see **Table 1**).

Therefore, it is evident that yeasts are not only found in corn silage, but that in the case of corresponding clinical disorders, the grass silages in the ration should also be considered. In this study, slightly more than 40% of the samples examined had yeast contents that were higher than the usual recommended limit values.

In general, in the case of reduced performance or illnesses in the dairy herd, special attention should be paid to the basic feedstuffs (in addition to corn silage, especially grass silage) and these should be analysed accordingly.

In most cases of clinical disorders in cattle a causal relationship could be established between microbiological findings and pre-reported disorders (see **Table 2**).

Thus, deviations in the hygiene status could be found in every 5th sample examined (22–25%). On average, 25% of the samples already showed deviations in the sensory test. Therefore, it makes sense to take a critical look at the basic feed from time to time.

	Number of samples (n)	Relative proportion (%)
Aerobic bacteria		
Tolerable levels (< 2 x 10^5 cfu/g*)	82	75
Non-tolerable levels (> 2 x 10^5 cfu/g)	27	25
Moulds		
Tolerable levels (< 5 x 10^3 cfu/g)	96	88
Non-tolerable levels (> 5 x 10^3 cfu/g)	13	12
Yeasts		
Tolerable levels (< 2 x 10^5 cfu/g)	64	59
Non-tolerable levels (> 2 x 10^5 cfu/g)	45	41

**Fresh matter.*

Table 1
Microbiological quality of grass silages (n = 109) from practical farms in Germany.

	2017		2018		2019		2020	
	n	%	n	%	n	%	n	%
Total grass silage sent in*.	64		63		80		63	
Deviations in hygiene status	15	23.4	16	25.4	19	23.4	14	22.2
Preliminary report								
Post-heating after silo opening	2	13.3	6	37.5	3	15.8	1	7.1
Deviations in the sensory test	4	26.7	4	25.0	3	15.8	4	28.6
Reduced feed intake/milk yield	2	13.3	2	12.5	6	31.5	5	35.7
Enteritis, diarrhoea	4	26.7	1	6.25	5	26.3	—	—
Mastitis, abortions	1	6.7	2	12.5	1	5.3	4	28.6
Metabolic disorders, liver diseases	2	13.3	1	6.25	1	5.3	—	—

**Analyses due to clinical disorders, no control analyses.*

Table 2.
Quality of grass silages sent in to the institute (2017–2020).

In addition to the hygiene status, attention should also be paid to the botanical composition of the green fodder. The presence of velvet grass leads to a poorer acceptance of the forage and to a reduced regurgitation. While the number of chews per bolus averaged 250 ± 57 when hay is offered, the chewing frequency is significantly lower (146 ± 91 chews per bolus) due to the proportion of velvet grass (*H. lanatus*). On the one hand, this means that a smaller quantity of basic feed is consumed, which can lead to a loss of performance. At the same time, chewing and ruminating is reduced and less saliva is produced, so that the buffering effect is reduced.

Due to the more intensive nitrogen (N) fertilisation and the partly developing resistance to common herbicides, there has been an increasing trend in recent years towards the infestation of monocultures with poisonous plants [9]. Examples are the occurrence of black nightshade (*S. nigrum*) in maize crops [7, 9] or the dispersal of dog's mercury (*M. annua*) in intensively cultivated beet fields [10]. Whereas in past years illnesses of dairy cows as a result of ingestion of poisonous plants or plants with poisonous ingredients were rather the exception, recently more questions have been asked about this problem and there have been an increasing number of cases of poisoning.

This development can be explained, among other things, by altered management of agricultural land, such as the trend towards extensification (migration of poisonous plants from fallow land into cropland, restrictions of fertilisers, ban on herbicides). Renaturation measures (e.g. raising the groundwater level) can also promote the spread of certain poisonous plants (e.g. marsh horsetail). In addition, there are neophytes, i.e. plants that are not endemic to the respective area but are spreading more and more over time and as a result of climate change. For example, golden oat grass (Vit. D efficacy!), whose distribution was previously limited to the Alpine regions and southern Germany [11], is now also increasingly found in northern Germany. On the outskirts of cities, ruminants also come into contact with ornamental plants containing toxic substances that are not usually part of their food spectrum (e.g. planting hedges for privacy protection). In this context, the improper "recreational horticultural disposal" of ornamental plants with toxic ingredients on adjacent pastures should be mentioned [12].

Sometimes, under the assumption that detoxification processes generally take place in the rumen, feeds with a reduced hygiene status are used in ruminants, which is obsolete in other species (e.g. horses). The same applies to contamination of the feed with poisonous plants, although some can be quite lethal, these are used in ruminants under the assumption that they are detoxified in the rumen.

While in the years 2000 to 2005 only about 5 to 10 cases per year were sent in, in which feed was to be checked for the presence of poisonous plants or plants with poisonous ingredients, in the past two years there were already 87 (2019) and 117 cases (2020) in which the suspicion of possible contamination with poisonous plants was expressed in the preliminary report.

Offering a feed contaminated with dog's mercury resulted in severe clinical signs. This toxic plant contains mercurialin (= methylamine), trimethylamine, hermidine, saponin (1% in the herb, highest content at fruit ripeness) and essential oils. The main ingredient mercurialin, which belongs to the saponins, leads to liver damage and haemolysis in cattle (typical clinical sign: icterus). The animals typically show apathy, salivation, reduced rumen motility and are often laying in autoscultatory posture. Corresponding laboratory analyses show a high degree of haemolytic anaemia as well as haemoglobinuria [10].

The effect of mercurialin is also present in dried plant material, whereas no information is available on a possible influence of ensiling on the toxic ingredients. In order to clarify the extent to which the ensiling process leads to a degradation of the toxic ingredients, heifers (n = 6) were offered beet leaf silage contaminated with 20% dog's mercury in a feeding trial. Compared to the control group (n = 6), there was a significantly reduced basal feed intake (see **Figure 1**).

The presence of marsh horsetail in a total mixed ration TMR for dairy cows also leads to a significantly reduced feed intake (see **Figure 2**) and thus poorer milk production even at levels of only 1.25%.

In addition to the roughage, the composition of the milk performance feed is also significantly responsible for the performance of the dairy cow. Macroscopically noticeable deviations (also in comparison to the previous batch) upon delivery of the feed should be a reason to have not only the chemical but also the botanical composition of the feed checked. A check revealed considerable discrepancies between declared and actually found feed materials in almost 35%. In one clinical case report (reduced milk yield), a high proportion of rapeseed cake instead of rapeseed extraction meal led to a fat content in the milk performance feed of 7.5% (instead of the declared 3.5%). The use of this feed led to a fat content of over 1500 g/animal/d (tolerated limit: 800 g crude fat/cow/d) and thus to fermentation disorders in the rumen.

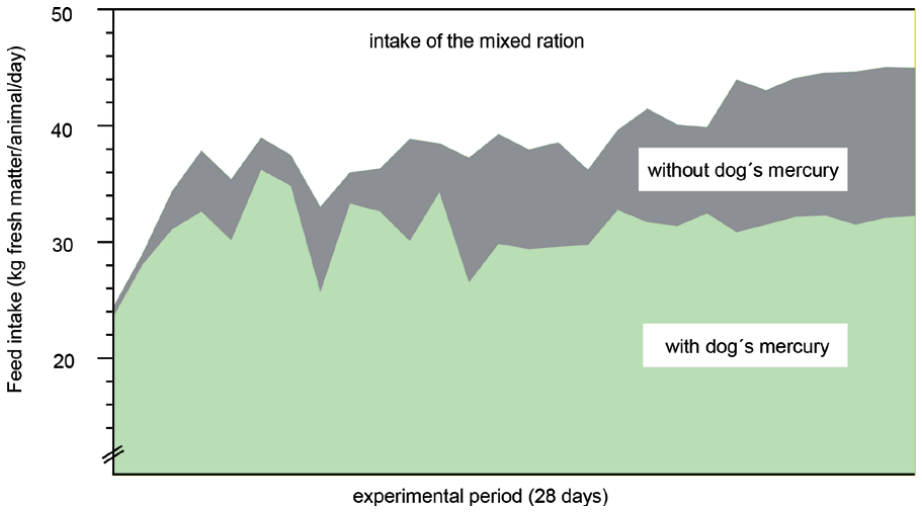

Figure 1.
Roughage intake of heifers feeding beet leaf silage with and without dog's mercury.

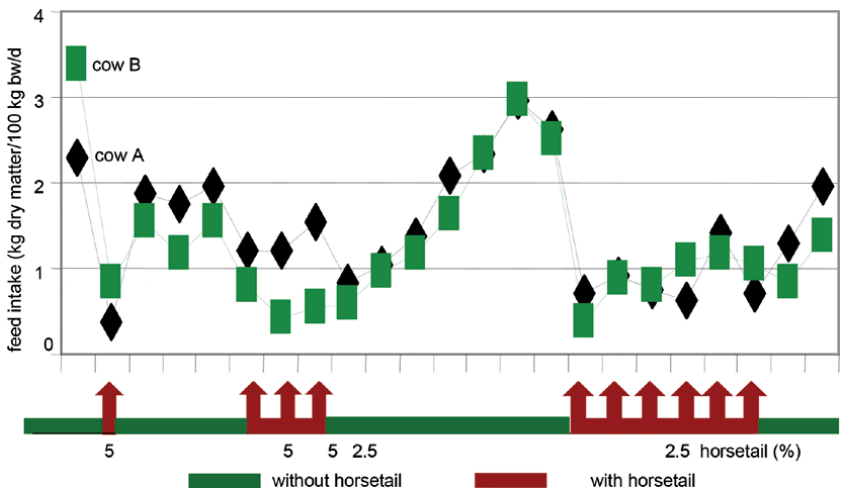

Figure 2.
DM intake of dairy cows when offered a TMR with different contents of marsh horsetail.

2.2 Meeting energy and nutrient requirement

The calculation of the ration by means of a computer program and table values must be preceded by an assessment (sensory testing, laboratory analyses), especially in the case of farm-produced feedstuff, as the nutrient content of feed can vary in a high range. This often results in discrepancies between calculated and actually fed rations, which give rise to complaints (see **Table 3**).

Often the rations had insufficient fibre content (< 16%) and at the same time very high starch and sugar content (in total > 300 g/kg dm). This feeding situation involves the risk of rumen acidosis. During the dry period, the animals were often oversupplied with energy. A possible fatty degeneration associated with this is undesirable, especially around the time of birth. In almost 20% of the samples, elevated mineral content was detected, which is contrary to the DCAB concept and involves the risk of milk fever.

	Checked rations (n)	Objections (%)
Lactation	87	
Crude fibre↓, Σ starch + sugar ↑	42	48.3
Crude fibre ↓, Σ starch + sugar ✓	11	12.6
Calcium ↑	13	14.9
Cu, Zn, Se ↓	10	11.5
Dry period	52	
Energy density↑ crude fibre↓	19	36.5
Minerals ↑ (DCAB)	10	19.2
Cu, Zn, Se ↓	5	9.6

Table 3.
Criticism of rations for cows during lactation and dry period.

Microbial fermentations during silage preparation not only influence the energy and nutrient intake of cows, but - depending on the amount and proportional composition - also milk yield and composition [13].

For the assessment of ensiling success and protein intake via fresh and preserved green fodder, the pure protein content provides important information (see **Table 4**).

If ensiling is only insufficiently successful, proteolytic processes can lead to protein degradation [14]. The result is reduced pure protein content, which - if only the crude protein content of the fresh and preserved green fodder is assessed - can lead to an incorrect assessment of the protein supply of the dairy cow (see **Table 5**).

Grass silages with a low true protein percentage in the total crude protein are supposed to contribute to the aetiology of a disease that is described as "factorial disease of dairy herds" or a higher incidence of fertility disorders, respectively [15].

During such proteolyses, biogenic amines are produced, among other things, which significantly influence the acceptance of a feed. Gamma-aminobutyric acid (GABA) is the so-called lead substance, the analytical detection of which can be carried out quickly and without great effort in the laboratory. GABA correlates closely with the pure protein level of a silage (see **Figure 3**) and thus gives a first impression of the silage quality. It is also known from feeding trials that GABA contents >7 g/kg dry matter lead to a massive reduction in feed intake [15, 16].

In the case of inhomogeneous mixtures (insufficient mixing, strongly inhomogeneous particle lengths of the individual ingredients), the cows can select more palatable components (e.g. maize silage), so that - despite a balanced ration calculation on paper - there can then be insufficient fibre and simultaneously higher starch/sugar intakes [17].

Feedstuff	n	Crude protein (% TS)	Pure protein (% of dry matter)	Pure protein portion of the crude protein (%)
Gras, fresh	40	21.3 ± 2.5	18.9 ± 2.4	87.5 ± 2.87[a]
Hay	36	14.2 ± 5.9	11.5 ± 3.9	82.3 ± 4.57[a]
Gras silage	186	17.4 ± 2.4	8.9 ± 1.2	51.1 ± 9.54[b]

Different lowercase letters indicate significant differences ($p < 0.05$) depending on the preservation method.

Table 4.
Crude protein and pure protein levels in fresh and preserved green fodder.

		High quality	Low quality
Dry matter	g/kg fm	375 ± 111	364 ± 108
Crude protein	g/kg fm	171 ± 20.8	180 ± 15.3
Pure protein	g/kg fm	94 ± 12.7	70 ± 7.3
Relation	% of crude protein	55 ± 6.1	39 ± 4.0

Table 5.
Pure protein content in grass silage of high or poor ensiling quality.*

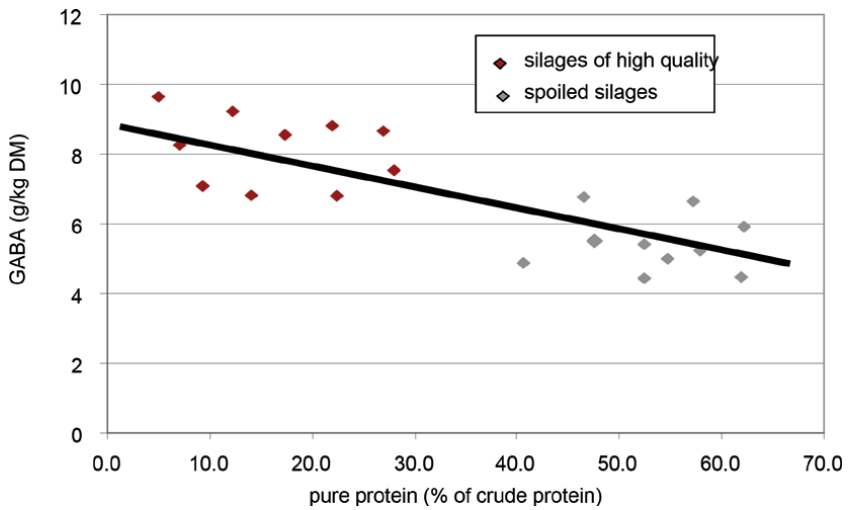

Figure 3.
GABA-levels in dependence of the pure protein level of grass silages.

A Total Mixed Ration (TMR) fulfils this requirement offering a simultaneous amount of richly-structured fibre and energy-rich components at the same time. But even if a homogenous and well-balanced feed mixture is offered the chemical composition (especially starch and fibre content) might differ between the offered and the actually ingested feed due to a selective feed intake behaviour in cows. In order to investigate this aspect 158 cows (Holstein Frisian) were split into two groups (group A, n = 76, TMR: 30.0 kg corn silage, 11.0 kg grass silage, 5.0 kg concentrate, 3.0 kg soybean meal, 2.4 kg alfalfa; Group B, n = 82, TMR: 29.0 kg corn silage, 11.0 kg grass silage, 3.6 kg concentrate, 2.0 kg soybean meal, 2.0 kg alfalfa). Starch and crude fibre were analysed using conventional methods. Measurement of particle size distribution followed recommendations of a commercial forage particle separator (Shaky 4.0, Wasserbauer, Waldneukirchen, Austria), consisting of three sieves with hole diameters of 19, 8 and 4 mm besides a collection tray for particles <4 mm.

Already in the first 30 minutes after feed offer a selective feed intake behaviour could be observed (see **Table 6**).

The distribution of particle sizes at the particular points of time as well as the chemical analysis of the feed suggest that the dairy cattle preferably ingest certain nutritious components (here probably corn silage). This selective feed intake behaviour implies the risk of SAARA due to the high starch and low fibre content in the actually ingested feed. The presented results show that selective feed intake is a non-negligible factor in dairy cattle nutrition. A synchronised intake of crude fibre and starch is not ensured even if the feed is offered homogenously. To avoid this

Group		n	Sieve analysis		Chemical composition	
			≥ 19 mm	< 4 mm	Crude fibre (% i. DM)	Starch (% i. DM)
A	Feed offer	5	34.7 ± 21.7	31.2 ± 8.38	21.5 ± 2.45	22.7 ± 1.57
	After 30 min	5	33.1 ± 4.95	29.1 ± 1.45	23.4 ± 0.92	19.9 ± 3.11
	After 7.5 h	5	54.5 ± 16.9	12.8 ± 2.46	26.7 ± 1.97	15.5 ± 1.57
B	Feed offer	5	25.7 ± 10.9	32.7 ± 2.12	24.0 ± 0.55	22.1 ± 3.76
	After 30 min	5	34.2 ± 14.9	27.4 ± 6.79	25.0 ± 1.10	18.7 ± 3.16
	After 7.5 h	5	51.7 ± 9.88	24.7 ± 9.19	25.7 ± 2.04	19.0 ± 3.69

Table 6.
Development of particle size distribution and crude and fibre level in a TMR in the period following feed offer.

selection all components would have to have identical particle sizes (as practiced with the compact-TMR or shredlage). The question is, however, if the cows would still show a physiological rumination behaviour under those conditions.

For decades, the question of the desirable/necessary supply of structured fibre to dairy cows (including cattle) has been the focus of animal nutrition science. Against this background, in 2014 the assessment of rations for dairy cows with regard to "fibre supply" was changed to a new system, namely the "physically effective NDF" (peNDF; [18]). The innovative feature of this concept is the unification of two criteria previously treated separately, namely.

- the physical form (length of fibres, size of particles, i.e. structure, determined in a sieve analysis) and
- the chemical composition of the total ration (sum of cell wall components, i.e. the NDF);

by multiplying the result of the sieve analysis (%, mass fractions) by the NDF content in the total ration (% of DM). According to previous studies and experience, a TMR should contain >18% peNDF>8 or > 32% peNDF>1.18 in the total DM to achieve optimal structural supply in dairy cows [19].

In feeding practice, the "shaker box" (so-called Penn State Particle Separator) offers an important tool for assessing the particle size distribution of a ration and, together with the NDF content, also for assessing the "structural effectiveness" of a ration. In our own investigations, however, it was shown that this approach is subject to various errors (**Figure 4**).

In addition to the mesh size of the sieves and the dry matter content of the silage (moist silage tends to stick together and thus gives the impression of longer particles), the result also depends to a large extent on the person carrying out the analysis. If an identical grass silage is examined by different persons, there is a very high scattering of the results.

2.3 Assessment of the feeding situation on the farm

A frequent animal welfare-relevant problem in the feeding of dairy cows is the insufficient intake of fibre-rich coarse feeds or the lack of synchronicity in the rumen. Consumption of sufficient quantities initially requires the production of

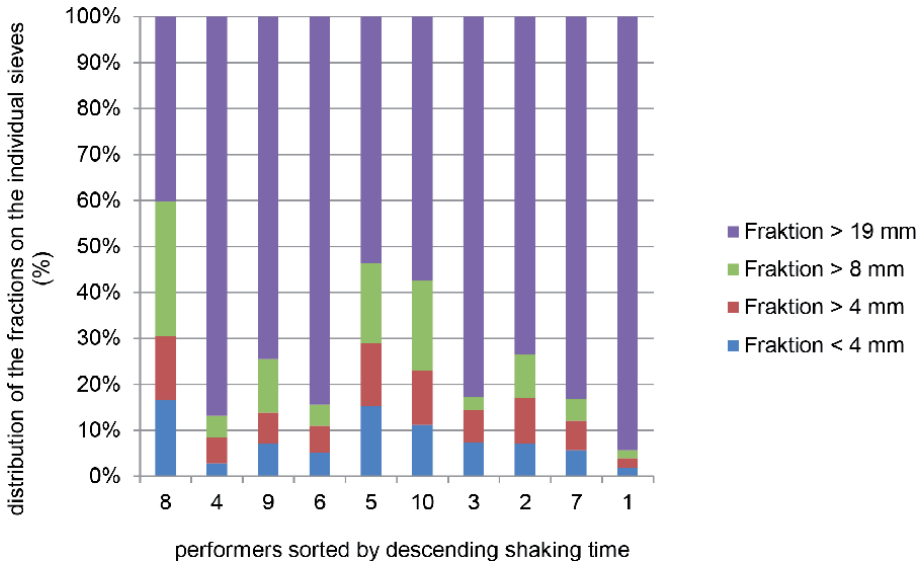

Figure 4.
Particle size distribution in a grass silage depending on the person carrying out the analyses.

high-quality grass silage and hay. However, studies from practice show that these often have higher contents of spoilage-indicating microorganisms (e.g. yeasts), sand, less palatable components (e.g. velvet grass) and ingredients (e.g. gamma-aminobutyric acid) or even just an unfavourable low dry matter (DM) content [7]. If these are mixed into a total mixed ration, the actually realised DM intake falls short of the expected one and the cow is sometimes not sufficiently supplied with energy and nutrients.

The factors influencing the DM intake of the dairy cow and thus ultimately also the performance are manifold and are summarised in the following **Table** 7.

A suitable indicator to check whether feeding is in line with animal welfare is the recording of feed intake quantities. Here the question arises as to whether the quantities of feed presented via the feed mixer and the quantities of concentrated feed correspond to the herd size. However, this approach considers the herd as a whole and not the individual animal. The same applies to the milk yield data, which

Feed	**Feeding**
DM content, structure	Adaptation (dry period)
Digestibility (Rfa)	Proportion of basic/fuel feed
Silage quality (fermentation process)	Quality (silage success)
Palatability	Availability of feed
Contamination	Water supply
Feed intake capacity	**Cow comfort**
Body weight, forestomach volume	Social stress, restlessness
Energy requirement	Climatic influences
Body fat content	Movement possibilities
Lactation status	Metabolic disorders

Table 7.
Factors influencing the dry matter intake of dairy cows.

provide conclusions on the supply of energy and nutrients (protein, fibre). Feedback from the slaughterhouse (e.g. increased incidence of fatty liver or liver abscesses) allows an assessment of the feeding situation on the farm, also under animal welfare aspects. For the assessment of the individual animal, the assessment of the Body Condition Score (BCS), which is also a component of the Animal Welfare Quality® Assessment protocol reviewed at PHÄNOMICS, is a suitable method. This procedure enables an assessment of the quality of the husbandry system from the animal's point of view. This would also satisfy another legal regulation of the Animal Welfare Act (§ 11, para. 8), which reads: "Anyone who keeps farm animals for commercial purposes must ensure through in-house inspections that the requirements of § 2 are met. In particular, he shall collect and evaluate appropriate animal-related characteristics (animal welfare indicators) for the purpose of his assessment that the requirements of § 2 are met." Thus, the livestock farmer cannot escape the responsibility to continuously check the feeding of his animals under animal welfare aspects (cross compliance; Directive 98/58 Annex No. 2; TierSchNutztV § 4 para. 1).

3. Conclusion

Feed and feeding conditions (especially ration design) are not infrequently the cause of health problems and/or performance losses in dairy herds. The possible damage (in terms of financial losses) ranges from reduced performance in the form of lower fertility, reduced milk yield or changes in milk quality to clinical disorders (coupled with treatment costs) and animal losses (e.g. sudden death of one or more animals). In contrast to the conditions in pig and poultry farming, where complete feed concepts are common, in dairy cow feeding the detection of weak points and failures, i.e. the causal clarification of feeding problems, is much more difficult. The variety of feeds and their variation in composition and quality, the differences in ration design depending on farm conditions (housing/feeding technique) and performance stage (dry period/high lactation) and, last but not least, the considerable individual variation in feed intake (concerning the quantity and ratio of various components of the ration) explain the particular challenge when it comes to possibly feed-related problems in dairy farms.

Satisfactory feeding practices require due diligence along the path from feed to food (´from stable to table´). However, ensuring these requirements and striving to avoid feeding-related problems in the dairy herd ultimately requires cooperation between the livestock farmer and the veterinarian.

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Chapter 12

Impact of Beef and Milk Sourced from Cattle Production on Global Food Security

Grace Opadoyin Tona

Abstract

Bovine meat and milk play a major role in the diet of humans and they have positive impact on global food security. The aim of this review work was to investigate the impact of bovine sources of meat and milk on food security in the low, medium and high income countries. Bovine source meat and milk could have impact on the nutritional, health, work, income, educational and recreational needs of humans. However, the feeding needs of bovine are mainly met with forage materials which do not compete with human foods. The beef and dairy cattle are raised mainly under the extensive system of production in the low and medium income countries, while the intensive system of production is that which is adopted majorly in the high income developed nations. The production of healthy beef and milk products may be observed to go a long way in preventing disease occurrence in both the cattle and the human consumers. The raising of fewer numbers of more genetically productive breeds of cattle under the intensive, semi-intensive and extensive systems of production could also have positive impact on global food security, sustainability and the mitigation of green house gas (GHG) emissions.

Keywords: bovine protein food sources, production systems, global food security

1. Introduction

Beef and milk sourced from cattle are important sources of protein in the human diet. Globally there is increasing demand for food, feed and fiber sources. Animal protein sources are usually found to be of higher cost and of high demand in the quest to solve and meet the demand for human food security. Bovine protein sources were described [1] to have high density of macro and micro nutrients per 100 g. Again, they were reported to contain essential nutrients difficult or impossible to find in other foods, they have micronutrients in biological forms that enhance their uptake into the body system (bioavailability). Furthermore, bovine protein sources were characterized to have high digestibility and high biological value of proteins with amino acid profile of essential and non essential amino acids that meet the human body system requirements [1]. The rearing of beef and dairy cattle is found to be of great importance in the promotion of food security strategies, as they serve as sources of nutrients dense foods, regular income and other benefits [2]. Some previous authors [3–5] have given the World Health Organization definition of food security as "When all people at all times have access to sufficient,

safe and nutritious food to maintain a healthy and active life". Yearly, more than enough food is produced world-wide to its entire population, yet food security remains unattained globally with hunger existing in many parts of the world, especially in the developing countries [5]. There exist a lot of wastage through the food supply chain from post-harvest losses to manufacturing and retail spoilage and thus directly threatening food security [5]. These problems are often found to cause increased global food prices, while there is very low purchasing power in several developing countries.

The three most common ruminant livestock are the cattle (bovine), sheep ((ovine) and goats (caprine), out of which bovine are the most prominent and the most predominantly and highly valued. This is evidenced by the fact that cattle meat (beef) and milk are the highest consumed world-wide, probably due to their large body size and weight, but low monetary value per animal. The bovine protein livestock is found to be of great importance in the promotion of food security strategies, as they serve as sources of nutrient dense foods, regular income and other benefits [2]. In a previous review work [3], it is reported that livestock production and marketing are very important to livelihoods of more than one billion poor people in Africa and Asia (which is one-seventh of humanity world-wide). These researchers also stated that beef production and marketing in West Africa provide sources of income to about 70 million people and dairy production supports about 124 million people in South Asia and 24 million people in the Eastern Africa. Another research finding [5] outlined that in a low income country (annual agricultural GDP of PPP$0.92 billion) livestock (particularly beef, dairy and draft cattle) ownership development increased income, raised the food security of those holding animals and altered the food environment of the people to enhance the diets of the livestock recipients' communities.

The challenge of food and nutrition security is reported to occur in both low and high income countries world-wide [3], though in different proportions and extents, similarly over-nutrition and over-weight do not exist only in the developed countries but also among few poor urban dwellers in underdeveloped countries of the world. This review is aimed to investigate the impact of beef and milk sourced from cattle production on global food security.

2. The contribution of bovine livestock production to global food security in the high, medium and low income countries

Cattle production could be practiced under the intensive, semi-intensive and extensive or range management systems. The beef and dairy cattle could also be managed under the mixed crop-livestock production system.

In high income countries, livestock keeping and production is mainly practiced under the intensive system and few farmers are involved [6]. Long term structures are used and the structures and building infrastructure are usually highly capital intensive. There is usually the use of exotic and high producing breeds of beef and dairy cattle. The production is usually defined as to either beef cattle production or dairy cattle production, and there is not a combination of these two. In the developed countries of the world, countries such as the United States of America, Holland and Argentina, production is highly specialized. Farming in the high income countries may involve land use regulations which may lead to high housing prices which are not affordable for the middle and low income countries households [7]. Economic opportunities for income generation intensive livestock production in the developed world usually involve a fewer number of people as compared to the larger numbers engaged in keeping livestock in the middle and low income nations [6]. Some

previous researchers [8] also pointed out that livestock keeping in all the three of high, middle and low income regions may be associated with other benefits related to leisure, recreation, tourism, education and inspirational opportunities.

In the low and middle income countries, majority of the households that keep livestock were reported [9] to have access to high consumption of livestock derived food such as bovine meat and milk than others who were not involved in livestock farming. In some of the low and medium income countries, about 40% of the livestock derived food was found to be obtained through importation from other countries rather than being sourced locally [9]. However, there is the need to continue to increase the local and global livestock production sectors, and also to have the plan to develop the small holder livestock production inclusive policies [9].

In the low and middle income nations, the international trade markets offer alternative means of meeting the nutritional need of the populace. Trade based strategies such as cross-border food supply networks are employed. Also, the net-exporting countries have been known to step down their food export during the times of food scarcity and thus posing increased threat of food unavailability to the net-importers [10]. Thus, the increased production of livestock derived food such as beef and dairy products, alongside the importation of food products from high income countries could be found to be an adequate strategy to meeting up with the increasing food demand and food insecurity in the low and middle income nations [11].

3. The intensification of bovine protein livestock production and the adaptation of crop-livestock production systems

There was reported [5] an increasing global population from about 4.4 billion in 1980 to 6.1 billion in 2000 with a 2% yearly increases which is again projected to reach 9.7 billion by 2050 [5]. It is well known that enough food is produced globally to meet the nutritional need of the entire human population, however, this food supply is not within the reach of people in all continents of the world. This is found to be true especially as it concerns the poor populace in the rural areas in developing countries due to some socio-economic barriers, harsh climatic and environmental conditions [12] and these lead to various challenges in the global food security. Therefore, there is the need to outline few of the various steps or projects conducted to investigate some of the bovine livestock production systems adopted worldwide to enhance food security.

3.1 Food security impact of dairy cows and heifers: a field experiment in the Zambia

In a livestock field experiment carried out in the Zambia, the impact of dairy heifers and cows ownership on household income and on household milk consumption was investigated [2]. The dairy heifers and cows were given out as pass-on-gift (POG) to 324 households and 2200 individuals over 5 different communities. Provision was also made for households and individual farmers to own dairy draft cows which also produced milk for sufficient milk consumption at home and some for local sales as source of income. The draft cattle were used for land cultivation and crop production. It was observed that this livestock development project increased incomes, raised the food security of those holding the animals and altered the food environment to enhance the diets of the cow recipients communities. Zambia was classified under the countries with an annual agricultural GDP of less than PPP$1 billion (PPP$0.92 billion). Also, Zambia was

classified as a region with historically low rates of large animal ownership and as a developing or low income country [2].

3.2 The economic and social impact of livestock production systems in the lower and higher income countries

Previous researchers [6] reported that livestock such as dairy cattle were a source of wealth in the lower-income nations and regions. They mentioned that there was a link between livestock ownership, household economic status and social welfare. These workers also stated that dairy production made significant contribution to poverty reduction at both the household and community levels in lower income nations. Dairy cattle ownership was also linked to income-generating activity for women [6]. The female dairy farmers in various lower-income regions such as India and Pondicherry were reported to have the ability to borrow money, obtain employment, have the provision of meat, milk and cow dung for manure or fertilizer, and also had the ability to use cows as draft animals to help reduce labour requirement on their farms.

However in the higher income countries such as in the US, there was stated found the intensification and consolidation within the livestock production sector [6], and these enhanced food and nutrition security.

3.3 The role of beef cattle production systems in food security

In a previous research [4], the importance of animal agriculture was stated to include the production of high quality proteins such as beef and milk for sustaining rural livelihoods and thereby contributing to food security. It was outlined that since the energy transformation efficiency in ruminants is very low, food security can be effectively promoted only if the major feeds given to ruminants (such as forage grasses and legumes) are not in competition with human food.

Beef and other beef products were further classified to possess the following qualities: They contain nutrients such as proteins and amino acids (essential amino acids particularly). These essential amino acids include leucine, isoleucine and valine needed for protein synthesis [13].

Some researchers [14] also mentioned that beef contain high amounts of glutamic acid, arginine, alanine and aspartic acid. Furthermore, protein ingestion from beef sources strongly increases muscle protein synthesis rates, and this effect was said to be due to the stimulatory effect of essential amino acids.

Beef was reported as a source of high quality protein and highly bio-available iron to enhance vitality in humans [15], and they contain lipids (polyunsaturated fatty acids – PUFA and saturated fatty acids – SFA).

3.3.1 Beef production under an intensive system (Argentinean perspective)

In Argentina, beef cattle are mainly raised on grazing lands, and thus the country is known to be a good producer of pasture-fed beef cattle, supplied with grains as energy supplement to bring about the production of pasture finished beef [4]. Beef cattle production in Argentina was reported to entail two major activities, which are: the cow-calf were kept on less productive or marginal lands; and the steer growing and fattening on more fertile soils [16]. These outlined pasture-finished beef is reported to be more likely to be leaner with lower cholesterol concentrations than feedlot beef [17]. The above described beef cattle production system is presented in **Figure 1**.

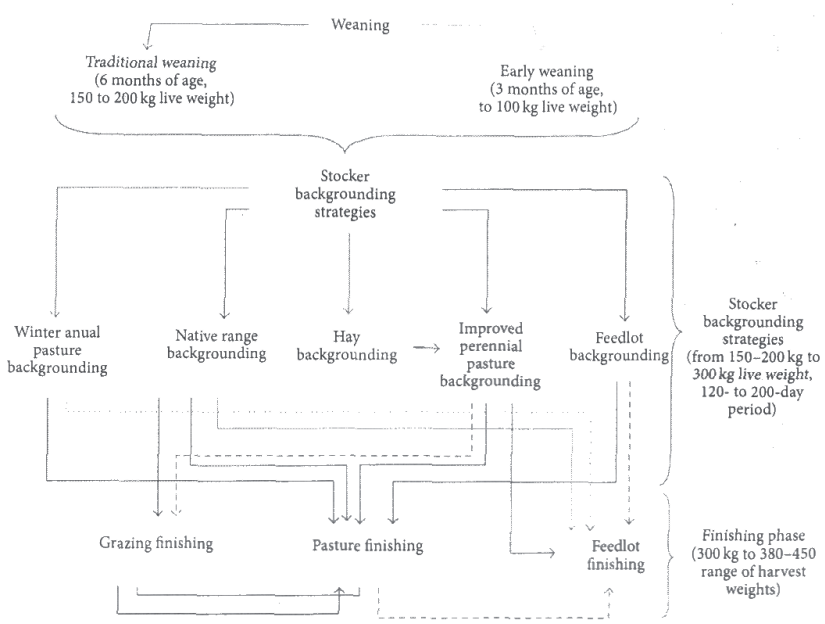

Figure 1.
The beef production system (Argentinean perspective). Source: [4].

Most beef cattle production (more than 70%) in Argentina is mainly carried out under the pasture (cultivated) finishing system (**Figure 1**) [4]. This system is least dependent on grain cropping feedlot but relies on adjusted forage chains depending on rainfall, environmental temperatures and soil quality. This system could however be practiced in rotation with grain cropping forage chains such as legume based pastures (principally alfalfa) and small-grain winter annual crops (rye, oats, rye grass and triticale [4]. Most of the cattle fattening farmers are known to make strategic use of energy supplement when necessary, such as cereal grains (corn and sorghum). These researchers [4] also pointed out that feedlots are more useful in terms of land occupation and judicious land use, but less useful as regards environmental impact, competition with human diets and meat safety. The beef obtained from pasture finished beef cattle were also observed to be leaner and lower in cholesterol concentrations [17]. Therefore, in Argentina, beef production practiced under the pasture finishing system was found to give an improvement of the nutritional value and quality characteristics of beef and improved beef healthiness and global food security.

3.4 The role of the traditional small-scale dairy production sector and its effect on food security (using the strength, weakness, opportunity and threat (SWOT) analysis

A research study [18] was conducted on the role of the small-scale dairy sector (SSDS) in Jordan as it affects food security. The study employed the use of a general survey questionnaire and a participatory rural appraisal survey (PRAS). In the results of the work, the dairy sector in Jordan was classified into small, medium and large scale farming. The small scale farm was graded to have the ownership of not more than 9 cows in order to make a positive impact on food security for the poor householders. In the area of feeding and nutrition, these householders (pastoralists) had the opportunity of using some of the harvested products from their farms for food supply for their families and sold the surplus for income. The PRAS was conducted among these pastoralists using the strength, weakness, opportunity and

	Strength	Weakness	Opportunity	Threat
Small-scale dairy sector	• Pastoralists by inheritance • Dairy cattle herding is a vital source of income, and supports household food security	• Livestock householders are helpless women • Women subjected to greater workload	• Food security • Provide meat and milk • Cash, manure, fibers, draft • Employment generation and security • Gives support for local products production	• Absence of sustainable practices and supporting policies • Lack of awareness of sustainability
Feeding and nutrition	• Source of food for peri-urban areas • Practice of mixed farming system	• Water shortage, food scarcity • Low feed importation and environmental impact	• Low cost culled cows and milk production • Livestock serve as source of cash • Provide support for local fodder and hydroponic fodder production	• Degradation of rangeland resources • Increases of feed prices
Animal health services	• Available veterinary and extension services and support	• No logistic support for disease control and sustainable veterinary services • No regulations for animal health • Lack of training, farmer empowerment	• Increasing milk production • Access to market • Reducing production cost	• Increasing cost of veterinary services and medications
Product processing	• Low processing cost • Added value to products	• Lack oftechnical knowledge and know-how	• Employment generation • There is consumer demand on increasing milk yield	• High cost of energy and technology • No governmental support
Product marketing	• Lower selling price • High demand on traditional products	• No pricing policy but monopolization • Indebtedness to middle men • No skill of handling and packaging	• Marketing in nearby markets • Direct sale of products	• Poor hygiene • Bad roads and market infrastructure

	Strength	Weakness	Opportunity	Threat
Policy and benefits	• Milk and milk products are full food for food security • Increasing poor's income • Generating jobs	• No governmental support of marketing or trading products • Lack of investment in major livestock's infrastructure (eg. Roads, water network, electricity)	• Good investment opportunity • There is increasing demand for milk and products	• No legislation for milk pricing • No hazard or risk supporting fund • High potential of being affected by climate change, natural disasters (eg. drought, storm, earthquake)

Source: [18, 19].

Table 1.
Strength, weakness, opportunity and threat of the small-scale dairy sector.

threat (SWOT) analysis. The observations made were outlined as strength, weakness, opportunity and threat as shown in the **Table 1**, which was however upgraded with findings from another similar research journal article [19].

The results summary was as follows:

The SSDS in Jordan was observed to positively impact food security for the poor householders. They consumed the milk produced from their cows and sold out the surplus milk as source of income.

The main strength observed from the SWOT analysis was that the householders were full-time pastoralists who practiced livestock keeping as their only source of livelihood. The major weakness was scarcity of feed or unavailability of feed resources which was due to water shortage or lack of rains. This in turn brought about increased feed prices, while the selling price of milk did not change, as this was dictated based on the selling price of the large scale dairy sector (LSDS). The greatest opportunity in the SSDS was that the farmers could obtain the training and awareness of technological inputs as regards their animals feeding requirements, milk handling processes, breeding and genetic resources information, disease control, post-harvest storage facilities and access to markets and marketing techniques, as well as policy support.

However, this was more applicable to the holders of exotic dairy cattle breeds. The threat faced was the high cost of milk production without the corresponding increase in their cattle meat and milk prices. It was reported [18] that prices of meat, milk and other products were dictated by the LSDS producers who received sufficient technology and policy support from the government. The government was found to focus more attention on the LSDS meat, milk holders since they were the main providers of meat and milk in the country.

In the concluding points on this research, it was stated that despite the threats and challenges faced by the SSDS producers, they could succeed by practicing the use of better genetic lactating dairy animals such as the exotic breeds of cattle, better animal feeding and milk processing techniques, better market access, by forming an association of SSDS farmers and by planting more fodder on-farm. These steps could assist the SSDS in Jordan to attain food security and poverty alleviation for the resource-poor farm holders.

In another research article, the outcomes of workshops conducted by the State of Palestine Ministry of Agriculture in partnership with FAO on the 28 January 2014 in Gaza, and on 12 February 2014 in Ramallah, the strength, weaknesses, opportunities and threats (SWOT) analysis of the ruminant livestock sectors were outlined [19]. One component of the strength stated was that dairy cattle herding is a vital source of income and supports household food security. The weaknesses outlined included the fact that women were subjected to greater workload than the men. Other weaknesses were as follows: lack of training, extension services and farmer empowerment. In terms of policy and benefits, the weaknesses reported were the lack of the government's investment in major livestock infrastructures such as roads, water networks and electricity. One of the opportunities mentioned was the provision of support for local products production such as the cultivation of local fodder and hydroponic fodder. One of the threats outlined was the high potential of the State of Palestine livestock sector being affected by climate change and natural disasters such as storm, earthquake and drought.

4. Some suggested solutions for the elimination of problems of human diseases associated with the consumption of beef and milk from bovine sources

Livestock such as cattle could cause negative impact on food security by transmitting diseases to humans through the consumption of contaminated meat, milk

and by-products [3]. These diseases could limit peoples' ability to work and earn income to meet their needs. The intake of livestock foods and by-products could also cause some harm to the nutrition and health of humans through the transmission of zoonotic diseases such as listeriosis and toxoplasmosis. These diseases were stated to cause about 2.2 million deaths a year, mostly among the low and middle-income countries [20]. In a research [1] reported that the global burden of food borne diseases resulted from 31 hazards which were considered in 2010 to result to 33 million disability adjusted years (DALYs), and that 98% of this burden fell within the low and middle-income countries. In view of these afore mentioned, efforts to promote the consumption of livestock derived foods such as beef, milk and by-products should go along with suggested solutions to improve and assure food safety [1].

4.1 Practical solutions to human health threats

Practical solutions to human health threats from livestock especially in the developing countries, where there are small scale producer demands collaborations between veterinary and public health researchers and officials such as meat inspectors [21].

i. Overconsumption of animal-source foods can lead to ill-health and affect human well-being, thus causing harm to the individuals, households and impacting whole societies [3].

ii. Livestock sourced foods such as fatty red meats and hard cheeses could cause cardiovascular disease. Similarly, processed meats such as bacon and ham have been associated with the risk of contacting pancreatic cancer [22].

iii. Animal-source foods, particularly the processed food should not be over-consumed but eaten occasionally and in small amounts.

4.2 Management practices that could be adopted in the dairy industries

i. The adoption of practices that reduce microbial contamination should be emphasized [23].

ii. Microbial contamination of milk should be minimized by the observation of hygienic standards that can be easily evaluated. Microbial counts of livestock foods produced should be checked at defined time intervals.

iii. The diagnosis of salmonellosis or listeriosis on dairy farms should be regarded as indications that other potentially infected animals may be present in the herd.

iv. Coliform counts on milk bulk tanks should be routinely carried out and minimum standards of coliform counts should be aimed at. Presence of coliform in milk is an indication of fecal contamination.

v. A reduction in the national regulatory limit for somatic cells count in milk bulk tanks should be considered based on standard milk safety limits.

vi. Raw milk harvested from dairy farms should be pasteurized to destroy pathogenic organisms which are risk factors for food borne diseases.

vii. Inappropriate use of anti-microbial agents should be minimized to prevent the development of antimicrobial resistance in animal/livestock pathogens.

viii. The presence of high somatic cell counts in milk is an indication of poor hygienic practice. Also, the presence antibiotic residues in milk beyond the minimum standard limits could be harmful to both the well-being of the dairy animals and in humans.

ix. Change from the adoption of the hazard analysis critical control point (HACCP) program to hurdle technology in food processing plants: The use of HACCP programs were designed for use in food processing plants [24], and it was not very much accepted for use on dairy farms as it involved the review of existing management processes, the establishment of limits through the identification of critical control points, the use of routine surveillance procedures, effective record keeping, documentation of standard processes and other non-competencies in its use. On the contrary, the hurdle technology described by some researchers [25, 26] was embraced for use to replace the HACCP program. Hurdle technology involve the application of a combination of some selected 'hurdles' or steps to examining microbiological growth in combination with the processing steps that maintain and improve the microbial stability and sensory quality of foods [22].

4.3 Ensuring the production of beef and milk products that are free of antibiotic residues

In the developed countries such as the USA, most dairy farmers do accept responsibility for the safety of the milk and beef produced from their farms [22]. The linkage between farm production practices and the quality of processed products could however be weak.

In order to ensure the production of beef and milk products that are free of antibiotic residues, the beef and dairy cattle farmers should adhere to the antibiotic drugs withdrawal times as specified on the label use of the drug manufacturer [27].

Again, meat and milk samples should be collected from individual the beef and dairy animals and also from the milk bulk tanks and tested for presence of antibiotic residues. The observation of the antibiotic treatments withdrawal period specified is very important as this could dictate the need to discard milk or withhold the cattle from slaughter in order to ensure drug residues are below the determined maximum residue limit allowed by the food and drug administration (FDA) after an animal has received an antibiotic treatment [28]. These previous researchers [28] emphasized that it is important to ensure that meat, milk and milk products are of high quality, safe and free of antibiotic residues before being sent out for human consumption and these could go a long way in enhancing food safety and food security.

5. Contribution of beef and dairy cattle to green house gas (GHG) emissions, climate change and global food security

Climate change could be defined as the raising of temperatures, elevation of carbon dioxide levels and precipitation changes, which will all affect agriculture and food production [12] causing drought and increased temperature extremes in many food production areas world-wide [5]. This increasing global temperatures and extreme heat stress could cause a decline in global food production, food

availability, stability in food supplies and minimized access to food and food utilization [12]. There could be declining yield in major food crops such as maize and soybeans especially in the developing countries. As a response to address the challenge of emerging global climate change, the December 2015 UN Conference on Climate Change was held in Paris, France. There was an agreement adopted by 195 countries to implement the first universal climate agreement to combat climate change (COP21 2015). This agreement was set to limit global warming to less than 2°C as compared to the pre-industrial levels in the 21st Century. To reach this goal, it was estimated that global green house gas (GHG) emissions needs to be reduced by 40–70% by 2050 and carbon neutrality needs to be reached by the end of this century (COP21 2015). This could lead to an improvement in the sustainability of global food production.

Beef and dairy cattle production contributes to climate change through the emission of GHGs, and climate change could affect human health and well-being to a great extent [6]. The impact on human health occurs through morbidity and mortality from extreme climate events. The indirect outcomes of climate change effect could also occur through economic disruption loss of labour productivity, changing availability of food, water and materials [29].

Despite the above mentioned negative effects, how else could the raising of beef and dairy cattle contribute positively to climate change and impact on global food security? One of the ways to achieve this was stated as shifting to raising fewer and more productive animals, particularly ruminants of more productive breeds [3]. This would require enhanced access to breeding, animal health and higher ruminant feed production such as grasses, legumes, concentrate feeds and other inputs to keep such less hardy animals alive and productive. This could also lead to the attainment of lowered environmental temperatures [30] for the livestock. Another approach suggested world-wide is the promotion of tree planting or aggressive agro-forestry programmes in different countries, particularly in the tropics. The planting of forage legume browse plants such as *Gliricidiasepium*, *Laecaenaleucocephala*, *Sesbaniagrandiflora*, *Afzeliaafricana* could help to ameliorate the effect of climate change and at the same time provide fodder leaves for ruminants. Also, environmental preservation laws and policies could be enforced, particularly in the tropical low income countries such as Sub-Saharan Africa and Asia. There should be the encouragement of the formation of more forest reserves and the establishment of biological gardens in different countries in the world in order to minimize the destruction and extinction of valuable tree species. Beef and dairy cattle production could also have positive impact on climate change and global food security through the feeding of feed by-products, feedstuffs, feed ingredients and feed additives that result in the production of less methane gas into the atmosphere. In recent times, some researcher workers [31] observed that the inclusion of yeast (*Saccharomyces cerevisiae*) fermented polished rice or cassava root meal in a livestock diet could produce a feed supplement which could be used as concentrate diet for dairy ruminants. This feed supplement was found to have the capacity to modify rumen fermentation, lower methane production, which also resulted to improvement in growth rate and feed conversion.

6. Development of policies that enhance the benefits of economic growth

It is well reported that the economic growth of a country is crucial for there to occur poverty reduction [6], however, economic growth alone could be insufficient to bring about reduced poverty on a broader scale [32]. There is also the

need for favorable environment for entrepreneurial investment, lack of corruption and improved governance in the public and private sectors, in addition to having a transparent and accountable society [32]. Also the pursuit of poverty reduction needs to be made viable and achievable through being backed up with the development of policies that support the delivery of sound educational system and health services.

7. Future prospects of bovine protein livestock production impact on global food and nutrition security

Future prospects could be considered to direct livestock production, particularly the production of beef and dairy cattle towards the breeding of more productive breeds of livestock globally. This is equally required in the low and medium income countries as it is already happening in the well developed higher income countries. The production of more ruminant feed such as improved perennial pasture production to serve as baseline feed for ruminants should be continually made a priority and the natural grazing lands should be continually renovated into improved pasture lands. There should be continuous development of vaccination and medication programmes to keep ruminants healthy and more productive. The promotion of semi-intensive ruminant production system could be encouraged particularly in the low and medium income nations, so as to lead to realistic, 'easy-to-adopt' farming management practices that have potential to mitigate green house gas (GHG) emission for sustainability and the attainment of global food security.

8. Conclusion

Beef and milk sourced from cattle production was found to play a relevant role in global food security. Cattle meat and milk are found to be the most paramount in the supply of animal source protein in the diet of humans. They contain indispensable nutrients such as high quality proteins, essential and non-essential amino acids, highly bio-available iron and other mineral elements, poly unsaturated fatty acids that are needed for healthy living in humans, in children and pregnant women. Such nutrients may not be obtained in tangible quantities from the consumption of other ruminant livestock meat and milk. The beef and dairy cattle have high feed intake which is met based on the intake of fodder which are not in competition with the human diet.

This review work has shown that the global demand for bovine livestock food resources could only continue to be met through the raising of improved breeds of beef and dairy cattle world-wide. These animals should also be sustained not based on feeds that are in competition with human foods.

The specialized intensive system of production was adopted in the high income countries. However, varying ranges from the extensive to the semi-intensive were practiced within the medium and lower income countries. Despite the nutritional benefits derived from the consumption of bovine meat and milk, there is also the need to take cognizance of the reduction of any health risks in order to attain assured food safety.

There should also be the reduction of GHG emission from beef and dairy cattle production to meet up with the sustainability of the physical environment. There is need to put in place national and global development enabling policies needed to enhance and promote cattle farming practices for the attainment of global environment sustainability and food security now and in the future.

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Chapter 13

Assisted Reproductive Technologies as Veritable Tools for Improving Production Efficiencies of N'dama and Muturu Cattle Breeds in Nigeria-A Review

Cornelius Nwoga, Nnanna Ikeh, Matthew Onodugo, Paul Baiyeri and Ndubuisi Machebe

Abstract

Assisted reproductive technologies (ART) that have come to stay and are still being improved upon in developed countries are still in their infancy stage in developing countries like Nigeria. Nigeria's cattle population is estimated to be around 18.4 million. The number is far insufficient to meet the country's demand for meat, milk, and other cow products, let alone contribute to GDP. N'dama and Muturu are both Nigerian breeds that are resistant to trypanosomosis. They are humpless longhorn and humpless shorthorn types of beef cattle. The dairy and beef cow industries' inadequate adoption of ART is partly to blame for Nigeria's low cattle output. Sex determination, multiple-ovulation and embryo transfer (MOET), oestrus synchronization, artificial insemination (AI), in vitro fertilization (IVF), cloning, and genetic engineering are all examples of assisted reproductive technologies. It has been reported in humans, rodents and domestic animals, abnormal fetuses, newborns and adult offspring arise from ART. Improper matching of breeding animals mostly leads to overfat calves. This review centers on the applications and potentials of ART in the production of trypanotolerant N'dama and Muturu cattle breeds. Some unorthodox medicines which have proven effective in human reproduction can circumvent the shortfalls in the adoption of ART.

Keywords: assisted reproductive technologies, N'dama, Muturu, Trypanosomosis, Nigeria

1. Introduction

Assisted reproductive technologies (ART) have been successfully used to alleviate fertility issues in humans and to improve farm animal genetics. ART adoption has risen dramatically in recent years, and this trend is projected to continue [1].

ART has some drawbacks and restrictions. In vitro embryo production (IVEP), which was the predominant method for creating bovine embryos for transfer in 2017 [2], demonstrated reduced pregnancy rates after the transfer of in

vitro-produced (IVP) embryos when compared to natural breeding, AI, or even transfer of in vivo-derived embryos [3–5].

The use of IVEP in cattle is limited due to a high percentage of pregnancy losses [6, 7]. Despite improvements in IVEP procedures over the years, current studies have found greater early and late embryo mortality in IVEP-derived pregnancies when compared to in vivo approaches such as AI or multiple ovulation and embryo transfer (MOET) [8, 9]. Calving difficulty and abnormal birth weight [10], disturbed fetal development [11, 12] and epigenetic dysregulation [13] have also been reported recently.

Cattle production accounts for a greater proportion of the economy of commercial and semi-commercialized farmers in undeveloped countries globally. It sustains the economy of most developed countries through meat, milk and skin production. Cattle have as well been on the forefront of researches in biomedicine and reproduction. With the recent promotion in biotechnology, cattle have been improved for better production efficiencies.

The cattle population in Nigeria at present is 18,404,661 million [14] and an annual growth rate of 1.5 percent is being estimated in the herd. It is disturbing that although developing countries account for about two-thirds of the World Cattle Population, while the developed countries account for about two-thirds of total beef population [15]. The three predominant production systems and their contributions to the total population are extensive (82.1%), semi-intensive (16.8%) and intensive (1.1%). Cattle population by geographic zones reveals the percentages as follows: North-West (52%), North-East (27%), North-Central (19%) and South (2%) [16] as shown in **Figure 1**.

Indigenous breeds dominate the cattle industry in Nigeria and they primarily serve the purpose meat production and for savings as well as milk production. Foreign breeds like Holstein Friesian, Brown Swiss, Jersey and their crosses can only be found in more intensive, specialized dairy farms [17]. Agricultural Policy for Nigeria [18] advocated the upgrading of local breeds of animals through the use of exotic breeds to a level not exceeding 50 percent to maintain hybrid vigor. Sequel to this, there is a high cost of importing an exotic bull or cow coupled with the physiological processes the animal must undergo before leaving a temperate region to the tropical region. Such processes like acclimation, acclimatization and adaptation must be achieved in time and space for a successful importation of an exotic breed into Nigeria. The economic implication of such venture is enormous.

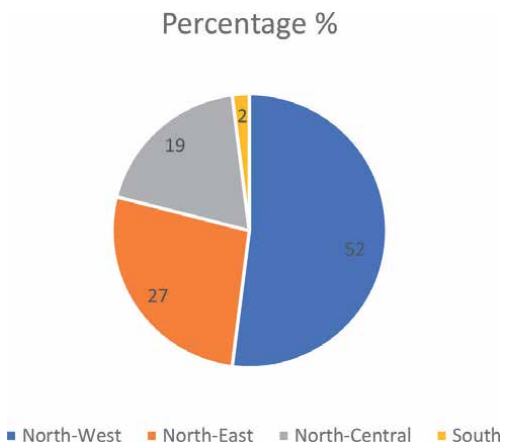

Figure 1.
Cattle population by geographic zones in Nigeria.

An easier approach to the above processes is the integration of ART into practices in the Nigerian cattle industry to boost production. There has been a significant increase in the utilization of ART, particularly AI and MOET, by developed and developing countries to produce millions of cattle. The adoption has led to a tremendous increase in both dairy and beef cattle production in several countries such as the United States and Brazil. In the national animal production research institute (NAPRI), Zaria, Nigeria, AI has been routinely performed since 1978.

Other interests include the monitoring of reproductive hormones and the improvement of oestrus synchronization and heat detection. Crossbreeding of the indigenous breeds of cattle with the exotic breeds is also on-going in an effort to upgrade their traits for beef production. Efforts have also been made to improve the milk productivity of indigenous cattle through crossbreeding with exotic cattle (Friesian) to produce crossbred cows (Friesian-Bunaji) with a genetic potential for increased milk yield per day [19].

Apart from NAPRI, AI is also performed in a few private commercial cattle farms. Unfortunately, these efforts impact a small proportion of cattle population in Nigeria. A recent study in northern Nigeria also revealed poor extension contact among dairy farmers, which blocks farmers from access to sources of improved dairy cattle technologies [20]. Tertiary institutions in Nigeria also present platforms for the utilization of ART in research that can improve animal reproduction and productivity. Many of these institutions have made considerable efforts in some areas of animal production, and in the treatment of reproductive diseases. Regrettably, there is a low potential for the application of ART, partly due to the absence of a number of equipment and facilities but also due to shortage of human skill or training [19] and the fear of the attendant consequences of embracing ART.

Clearly, an intensive application of ART will assist in improving reproductive efficiency and productivity in dairy and beef cattle farming in Nigeria through several approaches. Tertiary institutions and research institutes should be adequately funded and provided with modern research facilities, laboratories and equipment. Skills in ARTs should be incorporated and exploited in both teaching and research in animal science and veterinary medicine curriculums. There should be an increased synergy between farmers and researchers through agricultural extension services and above all, the farmers fears of post-ART consequences should be addressed through research.

The present turn of events in Nigeria demands that the discussion of the application of reproductive biotechnologies in cattle and their impact for future achievements be done.

The current discussion summarizes ART-based successes and implications in cattle breeding while also aiming to apply it to the effective enhancement of Nigerian dairy and beef herds.

2. Cattle breeds and distribution in Nigeria

2.1 White Fulani (Bunaji)

White Fulani ranks first among number and distribution in all the cattle breeds in Nigerian [21, 22]. It is estimated that it makes up roughly 37% of the national herd [23, 24]. They can be found in Nigeria's Western, Northern, and Middle Belt regions. They are completely missing in Borno, where Rahaji and Wadara abound, and in the south-east, where Zebu is scarce. Bunaji are said to be superior to all other Zebu breeds in terms of disease resistance and hardiness, which has favored their expansion into the derived savannah and the humid zone's edge [21, 22].

Late sexual maturity, a long interval between calving, and a short lactation period are some of the breed's drawbacks. The Bunaji, on the other hand, are known for their genetic aptitude to be sturdy, heat tolerant, and suited to local conditions [24]. A white coat color, a relatively large size, and a height of about 130 cm are all phenotypes. At maturity, bulls and cows weigh about 500 kg and 325 kg, respectively. They have a prominent hump, a little belly flab, and medium-length, upcurving, lyre-shaped horns. The White Fulani has three key economic characteristics: milk production capacities of around 2,300 kg per lactation, the ability to fatten for beef cum milk production, and the bull's proclivity to be utilized as a draught animal. At NAPRI-Shika, Zaria, offspring of White Fulani and Holstein crosses have resulted in improved milk production [24]. The average age for first calving was 42–45 months, however it might go as high as 5 years in Fulani herds. They contribute a large portion of the beef consumed in Nigeria [24, 25].

2.2 Red Bororo (Rahaji)

The Rahaji cattle herd is Nigeria's third largest, accounting for around 22% of the country's total herd. Except for a small population in southern Kaduna during the rainy season and an isolated population in the north-east Mambila Plateau, it is generally restricted to Nigeria's arid and semi-arid regions [21, 22]. The Red Bororo is one of the largest Zebu breeds, and it is distinguished by its rich red coat, enormous ears, and long, thick horns [26, 27]. The breed is adored by Fulani pastoralists who integrate it into their herds of 'white' cattle for crossbreeding purposes. It is adversely affected by poor nutrition and it is susceptible to humidity-related diseases [21]. Due to high mortality rates among the animals orchestrated by the movement of the herders down south into the Middle Belt, a Fulani clan, the Rahaji, who traditionally herded the breed and imprinted their name on it, has strikingly exchanged their stock for Bunaji [22].

2.3 Sokoto Gudali

According to [23], Gudali accounts for roughly 32% of the national herd. In Nigeria, there are two distinct forms of Gudali: the Sokoto Gudali (Bokolooji) and the Adamawa Gudali. The Bokoloji is found primarily in Nigeria's northwestern region, but it has recently spread throughout the country [25–27]. The Bokoloji is nearly hornless and has a homogeneous cream, light gray, or dun coloration. It has a lot of dewlap and skin wrinkles on it. The hair is short, and the skin is pigmented and thickIt has droopy ears, which milkers appreciate. At the National Animal Production Research Institute (NAPRI), Shika, the Sokoto Gudali beat the White Fulani in terms of milk yield [24, 25]. The calving interval is 360–450 days. The females have well developed udders with good teats which make them to be regarded as indigenous dairy breed. The average weights at maturity are 330 kg for the female and 450 kg for the male. The average milk production per lactation of the female is 1,500 kg [25].

2.4 Adamawa Gudali

As its name suggests, the Adamawa Gudali is only found in Adamawa [21, 22]. It is thought to account for roughly 2% of the national herd [23]. In Nigeria, two prominent local kinds are recognized: the Banyo, who have Rahaji blood and big horns, as well as a white face and red eye patches, and the Yola, who have a Muturu admixture [28]. Since the 1950s, the Muturu characteristics have gradually vanished, and local herders no longer identify the Yola breed as a separate variation.

Adamawa Gudali has a similar conformation to Bunaji. Its horns are normally pied and medium in length, and its size ranges from medium to giant. The coat can be white, black, red, or brown. The pendulous hump, however, is the most reliable distinguishing feature between Adamawa Gudali and Bunaji. Both Kanuri and Fulani pastoralists share ownership of the land. Kanuri and Fulani both have mixed Adamawa Gudali and Wadara herds, Bunaji or Rahaji. Many farmers consider Adamawa Gudali to be the traditional race of the region, where they work in the fields. They are normally fattened in the compound and brought to market when they become too large to pull a plow successfully [25, 29].

2.5 Wadara

Wadara cattle are another Nigerian breed. They are light, medium-sized, and dark-red, black, pied, or brown in color. They have short horns and a little upright hump, and account for approximately 6.6 percent of the national herd. They are Borno's "indigenous" cattle, which the Koyam and other pastoralists refer to as "our" cattle. In literature, the Wadara is most commonly referred to as 'Shuwa,' after the Shuwa Arabs who also herd them. The Ambala, a kindred white-coated breed from Chad, is frequently imported into Nigeria [21, 22].

2.6 Azawak

The Azawak is another breed found in Nigeria and is an indigenous cattle the Azawak valley North-East of Nigeria. It is distributed along its North-Western border. It has medium-length horns. The color variations range from red for Azawak in Niger Republic to fawn, white, brown, pied and black color for those that enter Nigeria. They represent about 0.7% of the national herd. Except for minute population kept by indigenous herders in Nigeria throughout the year, the majority is seasonally transhumant [23]. They are majorly distributed along the border North and West of Sokoto but scanty populations are found in the North-West of Borgu and the border between Sokoto and Katsina [21, 22].

2.7 Muturu

The Muturu is a breed of West African dwarf shorthorn cattle with a small body. It has a blocky shape with fine-boned, short limbs. The body is compact, without a hump, and has a broad head and a straight back. Their horns are relatively short and have a somewhat dished face. In South-Central Nigeria, the Muturu are predominantly black or black and white. They are usually black and white on the Jos Plateau, but they are noticeably larger than those found in the lowlands. Northern populations are made up of brown, red, or tawny animals. Muturu cattle have a very disjointed distribution within Nigeria, implying a gradual retreat of a once-widely distributed population [25, 30]. Blench et al. [30] has examined Muturu's management, productivity, history, and distribution. Due to insufficient maps of Muturu distribution, accurate estimates of their numbers are difficult to come by. Muturu cattle are widely spread and less apparent than Zebu cattle, and they are generally kept in stalls where they are fed. As a result, published population figures are inaccurate. Northern Muturu data is lacking, and their trypano-tolerance has not been assessed, making them ineligible for inclusion in estimations of 'trypano-tolerant' cattle. International Livestock Centre for Africa [31], who estimated 120,000 Muturu, should be compared to [32], who estimated 60,000 Muturu, or 0.7 percent of the national herd. Akinwumi and Ikpi [33] found 85,000 people in five southern states after conducting a poll. Nigerian National Livestock Resource Survey [23], the

first survey to take into account all of the populated islands, estimated a population of 115,000 in 1990 [15]. Isolated Muturu populations exist along the Republic of Cameroon border, extending into South-Eastern Borno and merging with Adamawa's Michika-Mubi area. In the dry savannah, small populations can be found in the Atlantika mountains, south-east of Yola, near Cham, east of Bauchi, and south-east of the Jos Plateau [21, 22]. Another Muturu group appears north of Tegina in the North-West, with widely varying coat colors, implying a link with the North-East populations. Muturu once inhabited much of southern Nigeria and the west bank of the Niger River, with its extinction in many parts occurring only recently. Either Keteku or Zebu have supplanted Muturu, or Muturu is no longer kept by communities. Muturu once inhabited much of southern Nigeria and the Niger Valley west of the river, but they have since vanished from many areas. Muturu has been replaced by Keteku or Zebu, or Muturu is no longer kept by communities.

2.8 Keteku

Blench et al. [30] investigated the distribution and productivity of Keteku cattle in greater depth. Due to an increasing amount of Zebu blood in 'Keteku' herds, purelines of Keteku have become difficult to come by in recent years. Zebu cattle are being used to replace village herds as Fulani pastoralists migrate southwards, invading territories traditionally restricted to trypano-tolerant stock. The progeny of a Zebu x Savannah Muturu hybrid found near Biu in southern Borno and published in the literature [28] is a good illustration of how the local Zebu gene pool has become dominant. The name Keteku for a certain animal may represent the owner's cultural background as well as the animal's genotype. According to [31], the population of Keteku in Nigeria is 180,000 people. Keteku is less common than previously thought, with a wide range of distribution. It's unlikely that there are 100,000 of any kind.

Keteku is a variant of Borgu and is a stabilized Muturu x Zebu hybrid that is also trypano-tolerant [28]. It is also known as Kataku, Ketari, Borgu, Borgawa, and Kaiama. The Muturu and Bunaji characteristics are combined in this breed, with white, gray, and black being the most common colors, with red and brown appearing on occasion. It has longer horns, a smaller hump, and shorter legs than Muturu and Bunaji. In Nigeria, the populations of Keteku herds are limited to a narrow band along the Benin Republic border in the region known as 'Borgu,' as well as areas near to settlements in Northern Yoruba land. Throughout this region, Keteku coexisted with West African dwarf shorthorn, both filling the same niche [21, 22]. Keteku is sometimes purchased as an investment stock in the Ondo area by farmers who value its combination of size and trypano-tolerance. Keteku cattle have traditionally been raised on breeding farms and dispersed as part of livestock extension programs. Keteku is kept in the Government Livestock Centre in Ado-Ekiti [21, 22]. Crossbreeding of Zebu and Muturu is popular in various West African countries. Despite the fact that the two types came into touch, there have been few 'new' crossings of Zebu and Muturu in Southern Nigeria. Incompatibility and religious strictures have been cited by farmers in the South East for not crossbreeding the duo. In the Jos Plateau, ethnic competitions between the livestock farmers and animal production considerations have been cited as reasons for continuing genetic separation [21, 22].

2.9 N'dama

N'dama cattle are indigenous to Senegambia and neighboring areas in West Africa [29, 30, 34]. In 1939, N'dama cattle were introduced into Nigeria from Guinea for research purposes due to their trypanotolerance and larger size than Muturu

cattle [30, 35]. N'dama cattle are humpless and have a medium-sized compact body with lyre-shaped black-tipped horns. The male has a large head and a small dewlap. Light brown N'dama are commonly brought into Nigeria, however black and pied N'dama are found in Guinea. They were sold to farmers and pastoralists in an attempt to boost local herds' resistance to trypanosomiasis. Outside of institutions, there are few pure N'dama due to herders crossing N'dama with Zebu, yet pockets of N'dama exist in Northern Yoruba region [21, 22].

2.10 Kuri

The Kuri is a humpless long-horn with a huge body [21, 22, 29]. Its exact historical origin is uncertain. Kuri horns are unique in that they are swollen and spongy, unlike those of any other breed. It is 1.5 meters tall and weighs up to 550 kilograms. In terms of size, it is one of the largest African cow breeds. Kuri cattle come in a variety of hues and have the capacity to sustain in semi-aquatic environments. The Kuri cattle population is primarily concentrated in the old Lake Chad basin particularly along the lake's eastern coasts. Kuri cattle can be found in Nigeria's Yobe valley and as far west as Gashagar. Kuri cattle are sent to Kano's North East district to be employed as traction animals. The breeds are crossed with the Zebu in Komadugu Yobe and are typically called Jetkoram in the literature [21, 22].

3. Herd size and productivity of cattle in Nigeria

3.1 Herd size

Only after several variables have been evaluated can the optimal herd size for an area and a population be estimated [34]. The notion of optimum herd size takes into account the current environmental conditions, the species biological capability (performance), herd management technique, resource utilization, and distribution in general principles [36]. The world's cattle population stands at 1,000,967,000 with the top ten producers being the India, Brazil, China, United States, European Union, Argentina, Australia, Russia, Mexico and Uruguay. The above data are based on the 2021 ranking of countries with the most cattle [37]. The average beef cow herds are 43.5 for United States [38], 69 Canada [39] and 41 for Nigeria [40]. Nigeria with an estimated cattle population of 18,404,661 million [14] and an annual growth rate of 1.5 percent could not feature among the first 17 countries as shown in **Table 1**.

However, the current paper is based on the literature evidence based on the assumptions in Nigeria. Cunnings [41] estimated the size of the Fulani cattle herd to be 100–150, while [42] estimated it to be 80–100. Another study [43] found that the average cattle herd size was 41, and that the majority of herders (46.4 percent) herded 41 to 60 cattle. The pastoralist herd size ranged from 16 to 69 animals per herd, according to a recent survey of pastoralist households in Zaria and surrounds by [34]. In the humid rainforest of Imo State, Nigeria, the majority of Fulani pastoralists (63.60 percent) maintained herd sizes of 41 to 70 heads, according to [43]. With a population of more than 170 million people, Nigeria requires a large number of cattle to meet its demand for cattle and cattle products. As long as more than 80% of the cattle population is in the hands of traditional herders, supply will not be able to meet demand. Cattle importation is thus practiced in order to bridge the deficit. The imported total was 5,142 heads per year in January 1996. In a study conducted by [44], the pastoralist's operational sizes were evaluated to determine the makeup of the herds in terms of the class of cattle—steers, lactating or non-lactating cows,

World			1,000,967,000
Rank	**Country**	**2021**	**% of World**
1	India	305,500,000	30.52%
2	Brazil	252,700,000	25.25%
3	China	95,620,000	9.55%
4	United States	93,595,000	9.35%
5	European Union	85,545,000	8.55%
6	Argentina	53,831,000	5.38%
7	Australia	23,217,000	2.32%
8	Russia	17,953,000	1.79%
9	Mexico	17,000,000	1.70%
10	Uruguay	11,946,000	1.19%
11	Canada	11,150,000	1.11%
12	New Zealand	10,063,000	1.01%
13	Egypt	7,850,000	0.78%
14	Belarus	4,300,000	0.43%
15	Japan	3,922,000	0.39%
16	Korea, South	3,744,000	0.38%
17	Ukraine	3,001,000	0.30%

Foreign Agricultural Service/United States Department of Agriculture [37].

Table 1.
Ranking of countries with the most cattle.

and calves. Small scale pastoralists (SSP), medium scale pastoralists (MSP), and large scale pastoralists (LSP) were the three kinds of pastoralists (LSP). According to the findings, the SSP had an average herd size of roughly 17 cattle, while the MSP and LSP had 32 and 73 cattle, respectively. Furthermore, the LSP had more lactating and non-lactating cows, as well as calves, than the SSP and MSP, whereas the MSP had the most steer in the herd [45]. The herd pattern in Zaria revealed a gender disparity, with more cows than bulls, with cows accounting for 60 to 75 percent of each herd on average. Keeping more cows than bulls is advantageous to pastoralists because a simulation of herd dynamics demonstrates that when female calves outnumber male calves in the kraal, the herd's maximum growth is achieved [36]. The herd size had a 50 percent preponderance of young animals, with females (35 percent) and males (35 percent) as the genders (15 percent). The breeding cows made up 49.1 percent of the herd, while the breeding bulls made up 6%. The number of breeding cows and young females in a herd impacts the profitability of any cattle operation to a large extent [46]. This helps to explain why the Fulani herd has such a high number of breeding cows and young females. Except for selected and retained breeding bulls, the young males, who had previously been plentiful, were sold out before to breeding to supplement the family's revenue. Because the mating ratio is usually 1 bull: 20 cows, keeping a large number of bulls in the herd is uneconomical [47]. The ratio of cows to young animals in the herd was practically equal, according to the data (0.98). The ratio of breeding bulls to young animals was low (0.14), implying that breeding bulls and cows were almost equal (0.15). The ratio of young males to young females was 0.42, indicating that young females outnumber young males [36].

3.2 Reproductive performance

Reproduction is the major determinant of profitability in a cattle enterprise. A cow needs to be re-bred in 80 to 85 days post calving to sustain a 365-day calving interval. Percentage of body fats in cows is directly linked to poor reproductive performance. Inadequate nutrition causes poor reproductive performance and researchers have discovered that for proper functioning of the reproductive system, a certain level of body fat must be attained. It becomes easier to develop more cost-effective a nutrition program if all of the farm's cows can be managed similarly. This is certainly relevant when a farm's entire herd of cows is maintained as a single herd, which is common in small production units [29]. All cows suffer from poor body condition at vital times due to year-round calving. Reduced income per cow, prolonged time before rebreeding, poorly conditioned calves at birth, low quality and scarce colostrum, decreased milk production, a high rate of dystocia, and lower calf weaning weights are all possible consequences. A longer interval between rebreeding and weaning will result in a younger, smaller calf at weaning the following year, resulting in lower profits if the animal is sold at weaning. Calves that were weak at birth may find it difficult to get reasonable amount of colostrums. This may give rise to high susceptibility to diseases, light weaning weights, decreased feedlot performance and poor carcass traits. As reported by researchers, there is clear evidence cows with a moderate body condition had a shorter delay between calving and initial estrus than cows with a bad body condition [29].

According to [44], 6–10 calving per cow per reproductive cycle is allowed by about 90.90% of pastoralists in Nigeria, while about 9.10% usually allow about 11–15 calving per cow. Therefore, majority of the aged cows are often slaughtered for sale in most abattoirs [48]. However, cattle production and breeding efficiency on Northern Nigerian grazing rangelands is low, especially during the dry season [49]. Cows, for example, have a two-year age at first calving and a two-year calving interval [50, 51], steers achieve slaughter weight between 24 and 30 months of age [52], and off-take rates range from 2 to 10% per year [49]. The median age at first calving for the reproduction was 4.75 years, according to [34]. The findings corroborated previous data on Bunaji cattle herds gathered from the Jos Plateau [52]. However, the result was higher than 37-month reported at the National Veterinary Research Institute, Vom, a government farm [53]. The difference is due primarily to the dry season's low amount and quality of feed on grazing fields [50, 54]. In such circumstances, providing feed supplements to boost cow output may be recommended. However, before any nutritional modifications are proposed, it's critical to figure out which nutrients are restricting cattle productivity in a certain zone [55]. Akpa et al. [34] equally reported a before breeding, the average age was 4.05 years for bulls in the pastoral cattle herds. This may be probably occasioned by poor nutrition acting together with other environmental stressors. Ndlovu et al. [56] made findings that the age at which young bulls reach puberty is affected by nutrition and feed intake. However, [47] suggested that where controlled breeding is being practiced, young bulls of about 15 months of age should not run with the cows in the pasture. Some researchers have used standard technical coefficients to compare results obtained in research institutions such as the National Animal Production Research Institute in Zaria or the National Veterinary Research Institute in Vom, as well as those in Nigeria's traditional model [44]. Data were collected on reproductive performance and milk to butter ratio. The results, *inter alia* showed that the proportion of milk to butter was 1 liter to 100 grams. When compared to the data above, it can be seen that the calving cycle in Nigeria ranges from 29 to 43 months. The average age at first calving is 30 to 42 months, with a productive life of between 9 and 14 years [44].

3.3 Productivity

The most important nutrients influencing milk and beef production in semi-arid environments are protein, energy, and minerals [57]. Some studies have revealed that energy and minerals are not the limiting requirements for grazing cattle, but rather protein deficits are the cause of cattle productivity losses [58, 59]. Blezinger et al. [57, 60] however, reported that rangelands fail to supply energy and minerals in adequate quantity during the early to mid-wet season. The consequence is a retarded growth rate of cattle which turns out to be a main stumbling block to boosting body weight growth [61, 62] and, as a result, impacting semi-arid beef production In the semi-arid areas, rangeland energy and mineral supplies in the late wet and dry seasons are usually perceived as sufficient to support cattle production needs on pastoral systems [59, 60]. Thus, in community rangelands in semi-arid areas, cattle production efficiency is sometimes governed by nutrient availability, which is affected primarily by temperature and seasonal rainfall distribution [54]. The traditional cattle sector in Nigeria is characterized by low productivity due to seasonality of quantitative and qualitative feed shortages, which is arguably the most significant barrier to improving smallholder enterprise production and productivity [63, 64]. Permanent land damage is prevented through grazing on large expanse of land. The pastoralists use the approach to maximize spatial resource use by allowing soil rejuvenation. Negative consequences of seasonal fluctuations in feed supplies have not been adequately established on performance parameters of pasture cattle in the Guinea Savannah Zone of Nigeria. Such data is required for the development of effective feeding and disease prevention strategies. Cattle, for example, are susceptible to stomach discomfort due to a seasonal shift in food [64]. The changeover from a forage-based to a finishing diet strong in grain aids marbling in beef, but it also causes gastric distress. This may have a negative impact on their development. Similarly, seasonal variations in the quantity and quality of feed supplies have an impact on beef cattle performance and carcass quality [64, 65].

3.4 Assisted reproductive technologies in animal production

Assisted reproductive technologies (ART) are widely used in humans and animals in many parts of the world to expand our understanding of reproductive processes and to improve reproductive efficiency. Oestrus synchronization, artificial insemination (AI), multipleovulation and embryo transfer (MOET), in vitro fertilization (IVF), sex determination, cloning, and genetic engineering are some of the technologies used in animal production [66]. These are powerful technologies capable of enhancing productivity, and when combined with bioinformatics will provide more impact in the future of animal production [66]. The cow is typically monotocous with an average gestation length of 40 weeks and therefore a relatively long generation interval. The rate at which a highly desirable cow can be used to enhance the genetic state of a herd is slow if no interference is made [67]. Hence ARTs are particularly useful in this species because of the low reproductive rates and long generation intervals. In the cattle industry, ARTs were initially developed to increase the production of calves from parent cattle with high genetic potentials, but now offer many opportunities for beef and dairy cattle production.

3.4.1 Oestrus synchronization

Oestrous synchronization involves the application of pharmacologic means to control oestrus and ovulation in farm animals. As a result, female animals are forced to go through oestrus (ovulation) at a specified, opportune time rather than

when it would naturally occur. In general, the procedures rely on either artificially inducing premature luteolysis with luteolytic drugs (e.g. prostaglandin F2 alpha or its analogues) or temporarily suppressing ovarian function with progestagens.

Synchronization offers several advantages and facilitates the maximal and batch managements of AI and calving in cattle herds, thereby increasing productivity and decreasing costs in dairy and beef cattle production [68].

Synchronization may have some benefits in beef herds, such as decreasing the calving to conception gap, and hence the calving interval and possibly the calving season. Accurate detection of oestrus is critical to achieving high pregnancy rates particularly in large cattle herds. Hence oestrous synchronization offers another strategy to circumvent the critical problem of oestrus detection [69].

3.4.2 Artificial insemination (AI)

Artificial insemination has been utilized worldwide for more than 50 years. It is still the predominant technology applied for the improvement of reproductive efficiency and productivity in cattle through progeny testing and genetic improvement [67]. AI is the introduction of live spermatozoa into the genital tract of the female to cause fertilization by means other than natural mating. Semen from bulls can be extended and preserved at 4–5°C for a few days or frozen in plastic straws in liquid nitrogen at −196°C for years or decades. Semen from a few high-performance bulls can then be used to breed large number of cows leading to rapid genetic improvement and dissemination of new breeds within cattle populations [65].

Movement of preserved semen instead of live bulls would also improve trade, reduce production cost and also decrease the spread of cattle diseases usually transmitted by direct contact between cattle. The use of AI also prevents the rearing of bulls that involve added cost along with the possibility of causing injury or death to farmers or staff. Controlling and recording the time of AI helps to avoid indiscriminate mating (often observed in natural mating), thereby facilitating proper farm recordkeeping and fertility management.

3.4.3 Multiple ovulation and embryo transfer (MOET)

MOET was first proposed in 1987, and it demonstrated how MOET programs may increase genetic gains by raising selection intensity and shortening generation intervals [70]. Multiple-ovulation (superovulation) is a pharmacologic procedure that increases the number of oocytes released at ovulation by 2 to 10 times, hence raising the quantity of embryos that can be produced. Embryo transfer (ET), on the other hand, refers to the techniques used to collect embryos from a female (donor) and transfer them into the uterus of another female (recipient) where they develop to term. Typically, a cow ovulates a single oocyte during each reproductive cycle, and therefore may produce only 8 to 12 calves in her reproductive lifetime. However, utilizing the technology for MOET, it is possible to obtain 30 to 40 calves from a single cow over a period of a year [71]. Through MOET, the numbers of imported highly valuable and scarce cattle breeds could be multiplied rapidly, leading to increased genetic improvement of cattle populations [72]. Highly valued cows that are injured or too old to carry normal pregnancy could also be made to continue producing calves via MOET, rather than these animals being culled or sold for slaughter. Natural twinning ranges from 1 to 2% in beef cattle, but the efficiency of beef production could also be increased in intensively managed farms by inducing twinning using MOET. This technology also offers commercial advantage to farmers via a lower cost of importation of cryopreserved embryos compared to live cattle [68].

3.4.4 In vitro fertilization (IVF)

In vitro fertilization (IVF) is a technology via which fertilization and maturation of oocytes takes place outside of the female (in the laboratory). The method is also called *in vitro* embryo production. The resulting embryos are then transferred back to the same or different females for development. Mature oocytes can be collected by flushing the oviducts shortly after ovulation. Alternatively, immature oocytes can be obtained from abattoir ovaries or by aspiration of pre-ovulatory follicles using ovum pick up (OPU) from live cows. These oocytes must be cultured *in vitro* for 24 hours in sterile medium to allow for nuclear maturation prior to fertilization. Following *in vitro* maturation of oocytes, spermatozoa must also be capacitated using a capacitation medium (or alternatively by using ejaculated sperm) before they are capable of fertilizing the oocyte [66]. The technology offers the potential for large numbers of *in vitro* produced embryos together with exciting opportunities for other technologies in cattle reproduction such as sex determination, cloning, genetic engineering and embryo transfer.

3.4.5 Sex determination

This technology is useful when calves of a particular sex are considered to be more valuable than those of the opposite sex. For instance, dairy farmers would desire that the majority of their calves be female (replacement heifers for the milking herd) whereas beef farmers would prefer bull calves for their higher body mass and beef production potential. Sex could be determined either by semen sexing or embryo sexing. The presence of Y chromosome determines male offspring in mammals. In cattle, the X-bearing sperm contain 3.8% more DNA than the Y-bearing sperm. Thus, sperm can be separated using specific dye (Hoechst 33342) that binds to DNA and a flow cytometer/cell sorter. Embryos can also be sexed using several techniques including chromosome analysis (karyotyping), immunology, DNA analysis and detection of metabolic differences [66]. Sexed semen could be applied in farms to inseminate cows in order to create necessary sex calves, or to fertilize oocytes in vitro in order to produce required sex embryos. Sexed embryos could likewise be implanted into recipient cows to create sex-matched calves [73].

3.4.6 Cloning and nuclear transfer

These technologies involve cloning by embryo splitting to produce identical twins, triplets and quadruplets or the use of nuclear transfer to produce large numbers of genetically-identical or cloned cattle. In the nuclear transfer technique, the nuclei from either a blastomere (from early-stage embryos) or a somatic cell (other body cells) are fused individually to enucleated oocytes. The resulting zygotes are then cultured and transferred to recipient cows to develop till term. Interestingly, this technique has attracted much international attention since 1996 when the first mammal (the sheep, Dolly) was cloned [74] followed later by cloning in cattle [75]. With cloning technology, it is possible to exceed pregnancy rates of 100% in cattle farms. It also offers the potential for producing large numbers of genetically-superior cattle to drive increased dairy and beef production [69]. For instance, it normally requires 78 months to reach production flock status in cows, but this can be achieved within 33 months with the nuclear transfer technology [67]. The success rate for propagating animals by nuclear transfer is expected to increase along with a reduction in the cost as newer methods are developed in the technology.

3.4.7 Genetic engineering

Transgenic livestock (pigs and sheep) were produced for the first time in 1985 [76]. This technology involves transferring a selected gene into an embryo so that the resulting offspring carry and express that gene later in life. Animals that carry a copy of a desired foreign gene are referred to as being transgenic [69, 77]. Generally, transgenic technologies utilize embryo-mediated or cell-mediated genetic modification to generate an entire animal. In recent years, new technologies referred to as "gene editing" have also been added to the molecular tool box for genetic engineering of various organisms. Efficient and robust protocols are now available for producing sheep, goats, pigs, cattle and other species in which specific genes have been targeted for editing [78].

The technology has been applied to improve different aspects of animal production. In cattle, these include the enhancement of milk quality, muscle yield, disease resistance (mastitis, tuberculosis), or improved welfare such as the production of hornless dairy cattle [77, 79, 80]. Nevertheless, the application of genetic engineering in livestock production has been limited by several significant factors. These include the cost of large animals, long generation times, and most importantly, legal, ethical and public health concerns and considerations [78]. However, it is likely some of the newer technologies involving gene editing will become more acceptable particularly in the face of the increasing global animal protein demands and food insecurity. Already, the first genetically engineered salmon has received approval to be sold as food by regulatory agencies in the US and Canada [81]. It is likely that other international agencies will begin to reconsider regulatory gridlocks on animal products from genetic engineering. In Africa, and particularly Nigeria, genetic engineering of bovine embryos may offer opportunities for the production of cattle that retain the genetic predisposition to hardiness, adaptation to the tropical environment (e.g. heat stress) and tolerance to tropical diseases (e.g. trypanosomosis) while incorporating genetic potential for rapid growth and increased milk and beef production [82].

4. Main abnormalities observed as post-transfer consequences of bovine *in vitro*-produced (IVP) embryos in some breeds of cattle

4.1 Holstein x beef breeds

In Holstein x beef breeds, main abnormalities observed include problem of increased loss of embryos in Grade 2 IVP embryos, increased fetal size, increased fetal body weight in IVP, no placentomes in IVP [6]; increased birth weight, increased percentage dystocia, increased mortality in IVP in Holstein; Holstein x Angus cross-breeds [83]; increased birth weight and oversized calves at birth in IVP, increased percentage dystocia, increased heart weight at 13 months in IVP frozen in Simmental bulls [84]; increased percentage males, increased gestation length, increased percentage congenital malformations, increased birth weight, increased perinatal mortality and calving difficulty (all in IVP) in Holstein Friesian breed [85]; decreased pregnancy rates, increased percentage males, increased spontaneous abortion, increased birth weight, increased dystocia, increased calf mortality, increased abnormalities of the fetus and reduced intensity of labour in recipients (all in IVP) and removal of serum did not correct abnormalities in Holstein breed [3]; increased *IGF2* expression, increased dystocia and increased mortality in IVP in Holstein breed [86]; decreased fetal size in early pregnancy, decreased number of cotyledons, increased cotyledon size, increased birth weight in Angus; Angus x

Hereford cross-breeds [87] increased expression of genes (Heat shock protein family A (Hsp70) member 1A (HSP70.1), Sodium dimustase (Cu/Zn-SOD), Glucose transporter type 3 (GLUT3), Glucose transporter type43 (GLUT4), Basic fibroblast growth factor (bFGF), Insulin-like growth factor 1 receptor (IGF1R)) in blastocysts and increased calf birth weight in Holstein-Friesian breed [88].

5. Latching the lacuna

Technology adoption and transfer have been major problems in developing countries owing to so many reasons. Principal among them is lack of technical know-how, poor or near absence of funding, diversion of released funds for specific projects/corruption and weak agricultural extension agents who are either unaware of recent findings or who reluctantly refuse to diffuse information from research institutes to farmers.

ART in Nigeria should be given a linear but additive approach starting with AI which is the oldest and most pliable form of it. This can be combined with sperm sexing in the long run. Individuals and corporate bodies san train manpower on semen collection, estrous synchronization and artificial insemination. Reproductive physiologists should brace up for the challenge in this field.

Nigerian is blessed with tropical herbs which are used in unorthodox medicines by herbalists. Some of these herbs have proven to be potent in managing pregnancies and parturitions in humans. The same crude technology can be extended to N'dama and Muturu. In humans, pregnancies that exceed forty weeks without labour and parturition are managed with these herbs to prevent the fetus from becoming overfat so that labour and normal vaginal delivery will set in a short time. This will be averted if the gravid woman was placed on these herbs as a routine during the course of the pregnancy. These two breeds became animals of choice due to their resistance to trypanosomosis which has made it possible for them to thrive where other breeds have failed to thrive without inoculation against trypanosomosis. Pregnancy complications arising from using say Holstein Friesian semen to upgrade the duo will be managed with these tropical herbs which abound in Nigeria. This will open up new frontiers in research on cattle which may help to solve some of the problems outlined in Section 2.4 of this work.

6. Conclusions

It is worthy of note that the cattle population in Nigeria of above 18 million may appear false and misleading as it was not captured in the FAS/USDA 2021 ranking of countries with the most cattle. If the figure were true, Nigeria would have come displaced Russia to rank 8th in the list of countries.

Assisted reproductive technologies will not only bridge the gap in meat and milk consumption of Nigerians but it will open up a goldmine which will reduce the unemployment rate in this region. This technology undoubtedly will start in Nigeria and spread to neighboring countries in Africa.

Cattle production in Nigeria, which hitherto was business of the North will become a smooth running business in the South if N'dama and Muturu which have adapted to the more humid and disease-tolerant southern climate are improved upon using the ARTs. This will go a long way to improve the economy of Nigeria and equally mitigate the farmers-herders crisis which has consumed so many lives and properties. Above all, it well help Nigeria to attain self-sufficiency in meat production in the near future, become an exporter of beef and dairy products.

Conflict of interest

The authors do not declare any conflict of interest.

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Chapter 14

Promising Food Ingredients: Milk Proteins

Roua Lajnaf, Hamadi Attia and Mohamed Ali Ayadi

Abstract

Milk, well known for its nutritional properties, has also good functional properties as foaming, emulsifying and biological activities due to proteins. Milk proteins are then considered as promising food ingredients due to their particular structural characteristics leading to various interesting properties in the industrial field. Thus, the examination of the biological activities and techno-functional properties (foaming and emulsifying properties) of some milk protein fractions revealed interesting ingredients for food industry due to their nutritional value, which is of a great scientific and industrial relevance. This chapter presented an overview of the studied functional properties of some milk proteins.

Keywords: foam, emulsion, biological activities, caseins, whey proteins

1. Introduction

Milk proteins are known by their spatial structure and physical properties which can explain their use in various techno-functional properties (such as water absorption, emulsifying or foaming properties) in their native state or after a suitable treatment (enzymatic, physical or chemical treatments) [1, 2]. Overall, to have interesting foaming or emulsifying properties, proteins should be soluble, amphiphilic and tensioactive with the ability to orient and change the conformation easily at the created interfaces (**Figure 1**) [3].

2. Techno-functional properties of milk proteins

2.1 Foaming properties

Milk is well known by its important foaming properties encountered with many various milk-based aerated foods such as ice cream, cappuccino, whipped cream, chocolate mousse, etc. [4]. Indeed, milk proteins determine the structure and stability of milk foam and emulsions due to their particular physicochemical characteristics as well as their interaction with other milk constituents [4–6].

Foaming properties of milk proteins are attributed to their ability to:

1. absorb at the air-water interface leading to a rapid decrease of surface tension at the air-water interface

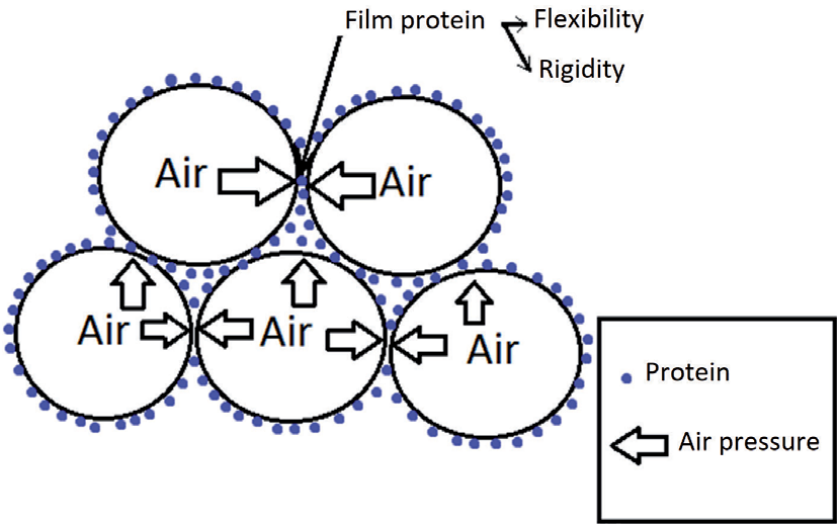

Figure 1.
Schematic presentation of protein based foam.

2. unfold at the interface with orientation of hydrophilic and hydrophobic groups of proteins at the aqueous and non-aqueous phases, respectively

3. form an interfacial film protein by using the interactions of partially denatured proteins to stabilize the created foam [7].

According to their structure and surface rheological properties, milk proteins can be classified in two main groups [8–10] flexible and globular proteins:

- Flexible caseins including proteins α_{S1}-, α_{S2}-, β- and κ-casein as well as the mixtures of calcium caseinates, sodium caseinates and acid caseins. They are flexible and have no tertiary structure.

- Globular proteins including β-lactoglobulin, α-lactalbumin, wheys obtained after cheese making as sweet and acid wheys. Overall, these proteins contain tertiary structure contrary to caseins, they are stabilized by disulfide bridges and preserve their globular molecular shape even after adsorption on the interface.

All milk proteins (β-casein, α-casein, κ-casein, β-lactoglobulin and α-lactalbumin) compete to the interface as follows: proteins with a more flexible structure such as β-casein are quickly adsorbed, whereas, globular proteins adsorb slowly [11]. Hence, the β-casein causes the creation of the foam due to its disordered structure. Indeed, it is considered as a "mobile" protein with an intrinsically unstructured molecular structure [12]. On the other hand, despite the low adsorption of whey globular proteins (β-lactoglobulin and α-lactalbumin), they intensively contribute to the formation of the protein film by improving its rigidity [13]. The order of the foaming efficiency of the insoluble and soluble protein fractions respectively of cow's milk is as follows: β casein> α casein = κ casein> whole casein, β-lactoglobulin> α-lactalbumin> whey [14].

Finally, whey globular proteins are characterized by a lower ability to adsorb at interfaces than those of caseins. On the other hand, their compact structure stabilized by the disulfide bridges, makes them suitable for creating a rigid interfacial protein film and consequently a higher ability to stabilize foams [8, 15].

The foamability of purified whey proteins is higher than that of the whole extracted whey. The β-lactoglobulin is the predominant adsorbed protein on the interface, regardless of its concentration ratio with α-lactalbumin [14]. At pH 6.7, this protein exists as dimers which are maintained by non-covalent interactions. Each monomer is characterized by two intramolecular disulfide bridges and a free thiol group. Upon adsorption at the interface, the β-lactoglobulin is not fully unfolded and its rate of lowering interfacial tension is slower compared to that of β-casein. However, once adsorbed, the created protein film of β-lactoglobulin is distinguished by a high density and an important protein–protein interaction in comparison with the protein layers of caseins. Indeed, the partial unfolding of β-lactoglobulin during its adsorption at the interface leads to the exposure of its free thiol group. Consequently, the adsorbed protein undergoes slow polymerization which is explained by the exchange between free thiol groups and disulfide bridges between the adsorbed β-lactoglobulin dimers [12, 16].

The purified β-lactoglobulin showed a better tensioactivity compared to other whey proteins such as the α-lactalbumin [17]. The β-lactoglobulin is characterized by significant foaming and stabilizing properties due to its high hydrophobicity and its unstructured conformation. On the other hand, the α-lactalbumin has interesting foaming properties but a low foaming stability [18]. This behavior is attributed to the compact globular structure of α-lactalbumin and the presence of four buried disulfide bridges which reduce its flexibility, and therefore its foaming and emulsifying properties [19].

Brooker et al. [20] showed that the main constituents of the milk foam interface are β-casein, β-lactoglobulin and α-lactalbumin. Other studies have shown that the stability of milk froth increases with increasing β-casein content [4, 10, 21, 22]. Indeed, during the creation of dairy foams, the β-casein is first adsorbed protein on the interface with a faster diffusion than that of globular whey proteins [23]. Thus, β-casein, once injected into a casein solution, is even able of moving other caseins such as α_{S1}-casein and β-lactoglobulin from the interface, while the reverse phenomenon is difficult to achieve (**Figure 2**) [25].

Thus, β-casein plays the key role in the stabilization of the foam due to its well-structured molecular conformation. It is even able to dissociate the α_{S1}-β complexes releasing the α_{S1}-casein and β-casein monomers. This behavior can be observed only at pH levels above 6, indeed at a pH close to 4.5, the solubility dominates foaming properties of caseins regardless of pH value [17].

Bovine proteins mixtures (β-casein-β-lactoglobulin and β-casein-α-lactalbumin) at different mixture ratios (100,0, 75:25; 50:50; 25:75; 0:100) presented an intermediate foaming behavior between those of pure β-casein and globular proteins alone (α-lactalbumin or β-lactoglobulin): the added β-casein increased significantly the foaming capacity value of protein solution. For β-casein-α-lactalbumin mixture, an increase of β-casein proportion from 25–75% of total protein amount, significantly increased foamability of 41%. For β-casein-β-lactoglobulin protein mixture, the foamability of the mixed systems was mainly dominated by β-casein. For instance, foaming capacity increased of 46.2% between pure β-lactoglobulin and the mixture

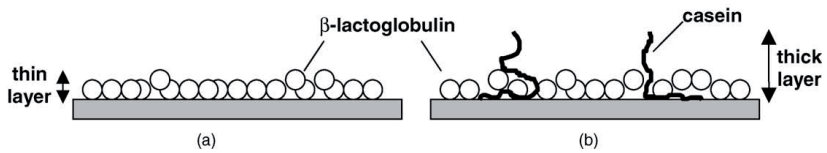

Figure 2.
The incorporation of casein in the structure of the β-lactoglobulin adsorbed layer; (a) monolayer of β-lactoglobulin; (b) incorporation of caseins into the β-lactoglobulin layer [24].

containing 50% of β-casein and 50% of β-lactoglobulin [10]. On the other hand, the foam stability is mainly governed by the β-casein regardless of the other mixed protein (β-lactoglobulin or α-lactalbumin). Indeed, the increase in the stability of foams is attributed to an increase in the diffusion and adsorption of milk proteins at the air-water interface [10, 26]. In the same way, Xiong et al. [27] studied foaming properties of caseins: whey proteins mixture at different ratios (80:20–75:25 and 80:20–40:60). These authors found that proteins at a ratio of 40:60 exhibited the lowest foam stability compared to that of 80: 20 sample because of the adsorption and spreading behavior of micellar caseins at the air-water interface, whereas, samples with ratios 80:20 and 75:25 did not show any significant difference in foaming properties [27].

Laleye et al. [28] reported that the milk origin and consequently the protein composition of whey have a great influence on its foaming and emulsifying properties. For instance, bovine and camel whey presented different foaming properties, which is attributed to the difference in protein composition of both wheys especially the absence of β-lactoglobulin in camel milk.

2.1.1 Effect of pH on foaming properties

Milk proteins molecules change their conformation and surface activity depending on pH level. Hence, foaming and interfacial properties also change depending on the physicochemical parameters of proteins [8]. For instance, foaming properties of skimmed milk decrease considerably at acidic pH (pH 4–5) because of caseins precipitation. However, these properties increase at pH 3 due to the dissociation of the casein micelles and the re-solubilized caseins characterized by a higher tensioactivity [23].

Surface properties of caseinates are predominantly determined by the β-casein regardless of pH value. Furthermore, surface pressure isotherms of caseinates were nearly identical to those of pure β-casein. Hence, caseinates adsorption layers were modeled by treating them as β-casein ones [8, 11]. The β-casein polypeptide is constituted of 209 amino acid residues; the first 50 are mainly hydrophilic, while the remaining 159 residues are mainly hydrophobic [29].

Neutron reflectivity studies [9, 30] have shown that the adsorbed β-casein layer can be represented as a dense inner layer adjacent to the interface with a thickness of 1–2.5 nm and another less dense outer layer released in the aqueous phase 3–7.5 nm in length. The inner layer includes the hydrophobic amino acids in a "train" configuration, while the outer layer is extended as a "tail" or "loop" constituting of hydrophilic amino acids. These data were used by Marinova et al. [8] in order to schematize sodium caseinates adsorption behavior at the air-water interface (**Figure 3a**). By reducing the pH to the pI (Isoelectric pH) of β-casein, the hydrophilic residues are electrically neutral at this pH value resulting a decrease the thickness of the protein layer (**Figure 3a**). Consequently, the decrease in the foaming properties of β-casein is caused by the precipitation proteins leading to a lower protein coverage of interface and a reduced electrostatic repulsion between protein films [8].

Unlike the foaming and interfacial properties of sodium caseinates, whey foams more at a pH levels close to the pI of β-lactoglobulin (pI = 5.2) and α-lactalbumin (pI = 4.1–4.8). At this pH value, the foam created by whey is more stable than that at neutral pH due to the reduced negative charge and electrostatic repulsion of proteins [5, 8, 23]. The modeling of whey protein adsorption layers is not realized by the major protein alone (β-lactoglobulin) as observed for sodium caseinates. Marinova et al. [8] represented the adsorbed layer of the whey protein mixture by an "average" of globular proteins which adsorb almost intact at the interface. At neutral pH, the molecule is negatively charged and electrostatic repulsions prevent the formation of a dense and continuous protein adsorption layer.

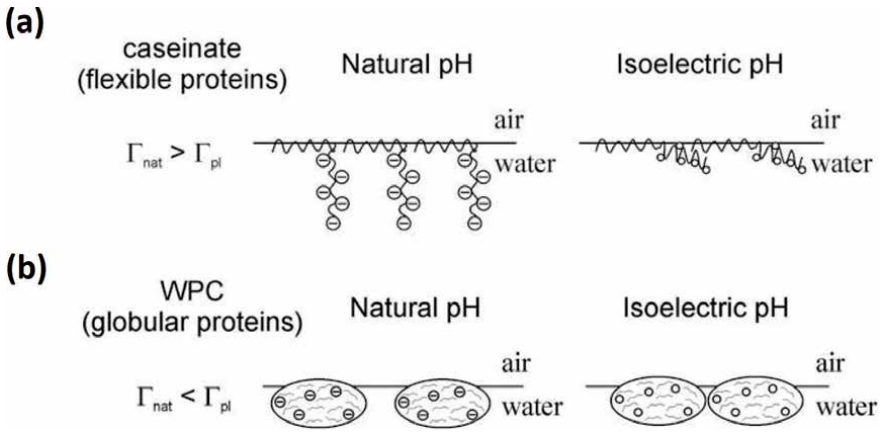

Figure 3.
Schematic presentation of caseinates (a) and WPC (whey protein concentrate) (b) at air-water surface at neutral pH (~7) and isoelectric pH (~4.5) [8].

However, in acidic conditions, the molecules are not charged and their adsorption and interaction are much higher (**Figure 3b**).

At pH 6,7, Lajnaf et al. [15] showed that the adsorbed protein layer of whey at the air-water interface consists of the β-lactoglobulin, while at pH 4.6, the adsorbed protein layer consists of the α-lactalbumin which is the most surface active protein in whey in acidic conditions. Indeed, the α-lactalbumin loses its bound calcium ion at pH values less than 5 and takes on the molten globular state and hence, becomes more surface active. However, the β-lactoglobulin is more rigid and thermodynamically stable at low pH levels leading to a less competitive adsorption of the protein in acidic conditions [23, 30–32].

2.1.2 Effect of temperature on foaming properties

Temperature is a very important parameter which affects the conformation of milk proteins and their distribution between both of whey and the colloidal phases of milk [33]. Therefore, temperature affects the molecular structure and foaming properties of milk proteins [33, 34].

Foaming properties of milk are significantly enhanced by increasing the temperature from 45–85°C, whereas stabilizing foam ability are maximum at 45°C [35]. After heating at 50°C, transmission electron microscopic observations shows that the film protein at the air-water interface consists mainly of the soluble caseins as well as whey proteins [4].

Overall, the denaturation of milk proteins after thermal treatments improves their foaming and interfacial properties due to their increased molecular flexibility, as well as their surface hydrophobicity [36]. However, foaming behavior heated milk proteins usually depends on the rate of protein aggregation. Denaturated and unaggregated proteins adsorb faster at the interface than aggregates, leading to the creation of foam. On the other hand, the adsorption of aggregates is slower, whereas, they contribute to the stability of the created foam (**Figure 4**) [37, 38].

Furthermore, greater foaming and stabilizing properties was measured for bovine milk proteins after increasing the temperature of thermal treatments, (up to 90°C for 30 min). This behavior was linked to the heat denaturation and aggregation of milk proteins especially globular whey proteins (β-lactoglobulin and α-lactalbumin), which led to an increase in the surface hydrophobicity and a decrease in the electronegative charge and interfacial tension [39].

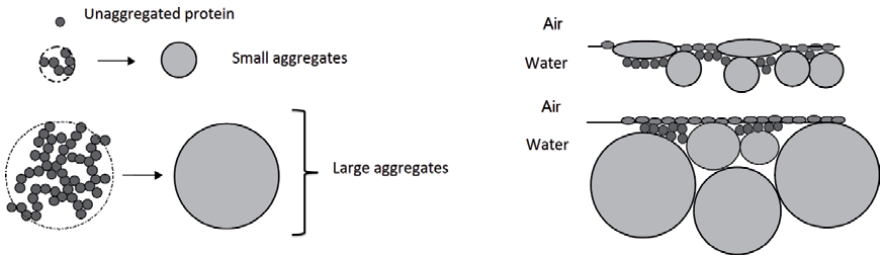

Figure 4.
Schematic representation of milk protein adsorbed layers adsorbed at the air-water interface by mixing unaggregated proteins and aggregates that within a heat treatment [38].

Similarly, whey proteins improve their foaming and stabilizing properties after heating process. However, the excessive heating denaturation of leads to a reduction of the resulted foam volume (for instance: 85°C for 750 s). Heating improves the tensioactive properties of α-lactalbumin and β-lactoglobulin by the exposure of the buried hydrophobic molecular parts of proteins leading to an improvement in their foaming and emulsifying properties [14].

2.2 Emulsifying properties

Emulsification is a common operation in food industry which is encountered with various food products such as mayonnaise sauces, soft drinks, salad dressings, soups, creams, butter and margarine [40]. Overall, an emulsion is obtained by mixing two immiscible liquids in the presence of one or more emulsifiers, where one is finely dispersed as droplets within another as oil in water emulsions (**Figure 5**) [16, 41]. During homogenization, emulsifiers are adsorbed onto the interfaces of freshly formed oil droplets leading to the reduction of the interfacial tension and oil droplets disruption. The most common emulsifiers used in the food industry are proteins which are the most surface-active agents in formulated emulsion systems [42].

During emulsion creation, mechanical shear is induced to create oil droplets within a continuous aqueous phase. Proteins dissolved onto this phase migrate to

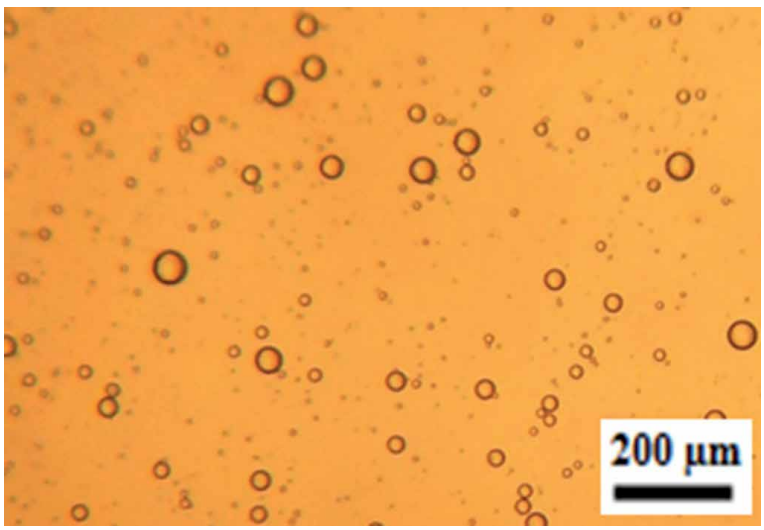

Figure 5.
Microscopic images of oil-in-water emulsions (85%) stabilized by whey protein isolate emulsion. The emulsion is diluted in a solution of SDS 0.1%.

the interface, and then realign to position its hydrophilic and hydrophobic amino acids towards water and oil phases, respectively. Once adsorbed, proteins accumulate to form a viscoelastic film around the created oil droplet and to keep the emulsion stable [41, 43].

Caseins are well known by their ability to adsorb rapidly at the oil–water interface, they are more effective in decreasing the interfacial tension than whey proteins. Furthermore, all casein types are adsorbed at the surface of oil droplet to provide stability to the resultant emulsion against coalescence and flocculation [31, 44]. Previous works evidenced that the diffusion and reorientation of β-casein at the interface occurs more rapidly than β-lactoglobulin and α-lactalbumin due to the low structuring of β-casein. Indeed, the β-casein is a flexible protein characterized by an amphiphilic nature allowing it to be the most effective in reducing surface tension at the oil–water when compared to β-lactoglobulin and even whole milk [12]. The β-casein is considered as a "disordered mobile protein" due to the low structuring molecular conformation and its rapid diffusion at the oil–water interface. It can occupy the majority of interfacial sites leading to a complete or partial replacement of the β-lactoglobulin molecules from the interface [45]. Seta et al. [45] noted that the protein mixtures containing different proportions of β-lactoglobulin and β-casein (1:3, 1:1 and 3:1) at pH 6.8 had an interfacial behavior similar to that of pure β-casein, suggesting the dominance of β-casein at the oil–water interface. Assessment of *in vitro* digestibility of milk protein isolate showed reduced emulsion stability compared with the intact proteins emulsions. Emulsion instability was hydrolytic enzyme preparation dependent and increased with increasing the degree of hydrolysis for a given enzyme [46].

2.2.1 Effect of pH on emulsifying properties

Emulsifying properties of milk proteins change significantly depending on pH level of proteins [41]. Mellema and Isenbart [47] studied the effect of acidification of a solution of reconstituted skim milk powder and whey protein on their interfacial properties (at a concentration of 0.7% (w/w)). These authors found that at pH 4.6, acidified casein micelles lose their colloidal stability, they aggregate and become less amphiphilic and tensioactive. Unlike the foaming and interfacial properties of sodium caseinates, whey proteins improve their flexibility when lowering pH level from 6.7 to 4.6. The dominant whey protein at the oil–water interface in acidic conditions is the α-lactalbumin: this protein adsorbs slowly at the interface but gives a high viscoelastic modulus [47]. The β-casein coated and stabilized the oil-droplets better at pH levels above neutrality when compared to acidic conditions. However, emulsions made camel β-casein at pH ~ 5 were unstable leading to significantly bigger oil droplets. Indeed, the acidification of caseins usually leads to the decrease in emulsion activity and stability because of precipitation and aggregation which alter their amphiphilic nature [42].

For whey proteins, Kilian et al. [48] compared their emulsifying behavior in both pH values 5.7 and 7.0. These authors reported that the emulsion was more stable in pH 5.7 than that at pH 7.0 with lower diameter droplets. However, the interfacial film formed by the proteins presented an essentially elastic behavior in both pH values with no significant differences in the resistance parameters of the oil–water layer interface [48]. Lam and Nickerson [19, 41] found that EAI (Emulsion Activity Index) as well as ESI (Emulsion Stability Index) values of whey protein isolate and the pure α-lactalbumin declined when pH increased from pH ~ 3 to pH ~ 5, before increasing at pH ~ 7. Stability of emulsions depends on the charge of the proteins: a higher stability is observed under conditions where electrostatic repulsion occurs. Indeed, electrostatic repulsion aided in keeping droplets from

flocculating. However, this behavior was less effective for neutrally charged protein near its pI [19]. Emulsifying properties of whey protein aggregates were also investigated. These fabricated aggregates (native, nanoparticles, and nanofibrils) showed significant emulsifying properties at pH 3 especially for whey nanofibrils. However, whey proteins nanoparticles had the highest EAI and ESI values at neutral pH [49].

The results of Lajnaf et al. [50] indicated that the α-lactalbumin molecules at neutral pH coated the oil-droplets better than those in acidic conditions with higher EAI values of apo bovine α-lactalbumin proteins (without calcium). Furthermore, ESI values of both apo and holo (with calcium) states of the α-lactalbumin were higher at pH ~ 7 than those at pH ~ 5. This behavior was explained by the electrostatic repulsive forces of the α-lactalbumin far from its pI which led to a better adsorption of the protein to the oil-droplet surface [50–52].

2.2.2 Effect of heating temperature on emulsifying properties

Structure–function relationships of heated milk proteins has been widely studied in the literature, especially as it relates to their aggregative properties after heating and nature of interactions (thiol-disulfide exchange reactions, hydrophobic interactions, and electrostatic interactions hydrogen bonding) [19, 39, 41, 53, 54]. These interactions can even alter the physicochemical and emulsifying properties of milk proteins molecules by heating the proteins to a partial or complete denaturation of the protein structure and to expose buried hydrophobic moieties [41].

The surface protein coverage of emulsions created with heated calcium caseinates solutions at 121°C for 15 min was higher compared to that of native caseinates. This behavior was attributed to protein aggregation upon heating and to the higher viscosity of the aqueous phase. On the other hand, milk proteins heating induces the increase in emulsion stability due to an increase in the diffusion and adsorption velocity of milk proteins at the interface and a decreased apparent viscosity [26, 44]. On the other hand, the emulsifying properties of whey protein were strongly associated with the size of generated thermo-induced aggregates [41]. For instance, heated whey proteins at 85°C and at pH 7 exhibited lower emulsifying compared to those heated at 55°C and 25°C. The difference in the size of the aggregates as a function of temperature: larger aggregates are usually obtained after heating at a higher temperature. Furthermore, Lam and Nickerson [41] found that EAI values of whey protein isolate were greater at both pH 3 and 7 since protein aggregates are smaller and the

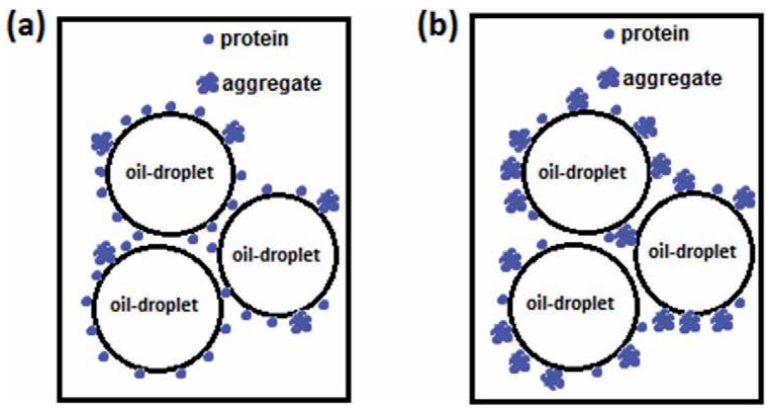

Figure 6.
Schematic presentation of whey protein based emulsion after a thermal treatment of proteins at neutral pH (a) and in acidic conditions (b).

hydrodynamic radii of the generated aggregates are lower leading to a rapid migration and integration of heated proteins into the interface (**Figure 6a**). In contrast, protein–protein aggregation was the highest after heating whey proteins at acid pH resulting a reduction in their EAI values. Indeed, the aggregation and hydrodynamic radii of the whey protein aggregates were highest in these conditions because of the reduction in electrostatic repulsion between heated proteins close to their pI (**Figure 6b**).

For pure whey proteins, the applied heat treatment to the α-lactalbumin at 65°C improves its stability to create and stabilize emulsions when compared to the unheated α-lactalbumin. However, increasing the temperature of the heat treatment from 65–95°C for 30 min leads to in reduction in its emulsifying stability because of the excessive denaturation of this protein [19].

3. Biological activities of milk proteins

3.1 Antioxidant activities

Overall, proteins have antioxidant activity through some amino acid residues such as cysteine, methionine and tryptophan. Indeed, these residues are involved in free radical-scavenging as they possess the highest antioxidant activity compared to the other amino acids [55]. Hence, the amino acid composition of proteins, their positioning and their accessibility are important in scavenging the free radicals [56].

Lactoferrin, representing between 1 and 2% of the total whey proteins, is characterized by its exceptional antioxidant capacity especially its ability to scavenge free radicals due to its sulfur-containing amino acids in its structure and the chelation of transition metals [57]. Native α-lactalbumin also exhibited significant antioxidant activities with respect to Ferric-reducing (FRAP), iron chelating and antiradical activities in both apo and holo forms with higher antioxidant activities for the apo form due to the greater exposure of antioxidant amino acids after calcium depletion [50]. Previous works indicated that caseins exhibited also important antioxidant activities. For instance, the β-casein samples showed significant iron chelating and antiradical activities depending on the protein concentration (0.1, 1 and 5 g/l) which could be explained by the higher content of antioxidant amino-acid residues in the β-casein protein [42].

Peptides generated from the enzymatic digestion of milk proteins are reported to have significant bioactivities such as antioxidant, antihypertensive, antidiabetic, immunomodulatory, antimicrobial, opioid properties. Indeed, peptides can be released through in vitro enzymatic hydrolysis, in vivo digestion approaches and fermentation, alone or in combination [58]. Antioxidant activities of native and hydrolyzed whey protein isolate were studied and compared to those of the major individual whey proteins (β-lactoglobulin, α-lactalbumin, serum albumin and lactoferrin) [59]. Antioxidant activities of whey proteins were significantly increased after enzymatic digestion compared with native proteins. The α-lactalbumin showed the highest FRAP (8.19 ± 1.19 μmol of Trolox equivalent/g) and ABTS free radical-scavenging activity (20.97 ± 1.44%) when compared of the other tested whey proteins with the release of the highest amount of the antioxidant peptides. These results lead to prefer the α-lactalbumin in food formulations to boost antioxidant defenses [59]. Investigations revealed that "Corolase PP", a commercial complex mixture of enzymes is the most appropriate enzyme in obtaining antioxidant hydrolysates from the pure α-lactalbumin [60]. The enzymatic hydrolysis of α-lactalbumin revealed a peptide having an IC50 inhibition value of 143 of superoxide radical-scavenging. This peptide was separated through a Sephadex G-200

column within a size-exclusion chromatography after peptic hydrolysis of whey filtrate [61, 62].

3.2 Antimicrobial activities

Except lactoferrin, the major milk proteins do not exhibit any antimicrobial activity in their native state even at high concentrations [63, 64].

Indeed, lactoferrin belongs to the protein family of transferrin family. It presented an activity against a wide spectrum of pathogenic microorganisms for humans. For instance, lactoferrin exerts a bacteriostatic and bactericidal effect on various Gram-negative bacteria such as *E. coli*, *Salmonella*, *Ps. aeuruginosa*, *H. pylori*, *Enterobacter*, *Yersinia*, *Porphyromonas gingivalis* and *Klebsiella pneumoniae*. Besides, this protein had antibacterial activities against Gram-positive bacteria such as *Listeria monocytogenes*, *Bacillus* and *S. aureus* [65]. Likewise, lactoferrin has been used against different yeasts such as *C. dubliniensis*, *C. albicans*, *C. glabrata*, and *Cryptococcus*, in synergy with different antifungal drugs [66]. In this context, several mechanisms of action of lactoferrin have been demonstrated against bacteria, fungi, parasites and viruses, including possible activity against the novel coronavirus SARS-CoV-2 infection [67].

On the contrary, pure β-casein and α-lactalbumin (apo and holo forms) had no bactericidal activity against *Escherichia coli, Staphylococcus aureus Enterococcus faecalis* and *Pseudomonas aeruginosa*. Furthermore, these proteins had no antifungal activity against, *Aspergillus tamarii, Aspergillus sclerotiorum Aspergillus protuberus* and *Penicillium bilaiae* even at a concentration of 5 g/l [42, 50]. However, pure proteins of milk from other mammalian species as goat and camel exhibited significant antimicrobial activities. For instance, apo camel α-lactalbumin showed moderate antimicrobial activities towards *Pseudomonas aeruginosa, Penicillium bilaiae, Aspergillus tamari* and *Aspergillus sclerotiorum* [50]. Furthermore, camel β-casein had strong antifungal activities against *Aspergillus tamarii* and *Aspergillus sclerotiorum* [42]. Meanwhile, the α_{S2}-casein from goat milk had antimicrobial effects against Gram-positive and Gram-negative bacteria, including *Bacillus cereus Escherichia coli*, *Listeria monocytogenes*, *Salmonella typhi*, *Staphylococcus aureus*, and *Shigella flexneri* [68].

The same trends were reported for native caseins which exhibited no antimicrobial activity: caseins just release bioactive peptides after digestion presenting these activities [69]. Once these peptides are released, they can act as regulatory compounds in the host organism with specific biological activities such as antioxidant and antimicrobial activities [70]. Similarly, four peptide fragments were yielded after a proteolytic digestion of the β-lactoglobulin by trypsin. These peptides exerted bactericidal activity against Gram-positive bacteria only [71]. However, generated peptides of β-lactoglobulin through the action of other enzymes such as alcalase, pepsin or trypsin, have been shown to be bacteriostatic against pathogenic strains of *E. coli*, *Bacillus subtilis* and *Staphylococcus aureus* [63].

On the other hand, previous works noted that the trypsin enzymatic treatment of α-lactalbumin led to the release of peptides with antibacterial activities. However, only one antibacterial peptide was generated after a treatment using the chymotrypsin enzyme. These peptides are known by their activity against Gram-positive bacteria, whereas, weaker effects were detected with Gram-negative bacteria. Overall, the peptides obtained from the α-lactalbumin after pepsin or trypsin treatments inhibited the growth of *E. coli*. However, pepsin treatment did not release any antibacterial peptides from the α-lactalbumin [63, 72].

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Section 5

Reproduction

Chapter 15

Chemical Signaling in Bovines: Understanding the Behavior and Way of Communication

Tawheed Ahmad Shafi, Md. Ferozoddin Siddiqui and Aejaz Ahmad Wani

Abstract

Chemical signals that mediate communication within animals of a species have been referred to as 'pheromone' a Greek word comprised of 'pheran' (means to transfer) and 'hormon' (to excite). These chemical messengers are transported outside the body and have a direct developmental effect on hormone levels and behavior, and therefore, have a potential role in modulating animal behavior and reproductive management. The sources of these chemical messengers are urine, vaginal secretions, feces, saliva, milk, sweat, breath and specialized cutaneous glands including the odor produced from hair and wool. After their release, are perceived through the olfactory system, eliciting both behavioral and endocrine responses characterized by profound effects on reproductive activity via the hypothalamic system that generates pulses of gonadotropin-releasing hormone. Their potential to transform the animal behavior and reproduction management has led to development and use of synthetic pheromones to manipulate estrous cycle, enhance estrous behavior, determination of time of estrus, and also facilitating collection of semen. Pheromones can act as a marker to detect estrus, diagnosing early pregnancy in farm animals and used for improving sexual desire. There is huge scope of application of pheromones once chemically synthesized and characterized, and would be of great interest to livestock owners and consumers. This chapter will discuss in detail the role of chemical signaling in shaping the behavior, reproduction and understanding the ways of communication in bovines.

Keywords: chemical signaling, pheromone, animal behavior and reproduction

1. Introduction

Lot of commonalities in chemical signaling have been observed between vertebrates (mammals) and invertebrates (insects), phylogenetically two distant taxa sharing common ancestors 550 million years back. Despite the fact, different taxa among vertebrates and invertebrates comprise of thousands of species, this commonality has been maintained, mainly due to selective constraints imposed by a terrestrial lifestyle that resulted in dominance of fewer animal phyla in terrestrial habitats. The dominance of Mammaliaformes has been attributed to nocturnal lifestyle that reduces the risk of getting predated from dominant archosaurs [1]. Similar trend has been seen in night active insects that were observed to be having

larger body sizes than day active insects sharing the same communities [2]. The nocturnal behavior may have resulted in decreased reliance on visual signals, and full reliance on chemical signals mediated by various mechanisms such as acquisition of hairs in mammals and insects that helped in dispersing chemical signals such as, tail hair tufts in Asian elephants [3], and hair pencils in male noctuid moths [4]. Also certain behavioral aspects are common in mammals and insects that are mediated by chemical signals, such as territory marking, or living in families, and recognition of individuals or group members [5]. Similarities have also been observed in signal processing pathways mediated by chemosensory proteins, olfaction and gustation that have evolved independently. In both the groups membranes of chemosensory cells are modified to increase surface area positioned in proximity to a number of accessory cells and these cells are bathed in liquid through which odorants travel to reach receptors [6]. Also neurons of booth the groups express same protein, synapsing in the same glomerulus, and olfactory cells project directly to the brain. However, olfactory receptor gene families and regulatory process of protein expression by neurons differ between mammals and insects [7]. Despite the organizational similarity in mammals and insects, differences have been observed in olfactory receptors and gustatory receptor proteins. In terms of taste, similarities have been observed in the organization of gustatory systems, recognizing nutritionally important dietary constituents such as bitter, carbon dioxide, water and sweet in insects, and bitter, salty, sour, sweet and umami in mammals [8]. In both mammals and insects chemicals involved in communication are secondary metabolites, derived from primary metabolic processes that show tremendous structural diversity due to differences in selective forces arising from different ecologies. Chemical signals involve primary metabolites such as amino acids, nucleotide bases, sugars, fatty acids and glycerol as starting materials that are utilized to produce secondary metabolites having conservative elemental composition mostly composed of carbon, oxygen, hydrogen, and nitrogen [9]. Chemical signals in terrestrial and aquatic environments differ significantly, and this structural variation has been attributed to the medium through which chemical signals travel. Diffusion in air is a function of molecular weight, that means lighter weight compounds will diffuse faster, such as most insect sex pheromones (MW 200–300) [10]. In contrast, diffusion of chemical signals in aqueous environment depends on water solubility (independent on molecular weight), conducive for signal transmission of biologically important large polar molecules such as proteins [10].

The term Pheromone was coined by karlson and Luscher in 1959 and the name of first pheromone extracted from Honey Bee was proposed as *Bombykal* [11]. Pheromone is a biologically active substance like hormone, a chemical substance secreted externally in urine, feces, or by sub-cutaneous glands or other biological secretions that cause specific reaction in a receiving animal. Exteroceptive cues playing a role in male and female interaction include olfactory, visual, auditory and tactic stimuli, that trigger a specific behavior or physiological change in recipient's endocrine or reproductive system [12]. Extensive studies in insects, rodents, swine, sheep, goats and cattle have established the strong influence exerted by the pheromones secreted by the male on reproductive activity in the female. It has been demonstrated that the urine of male mice, rats, feral species and other wild rodents contains a priming pheromone that is responsible for hastening puberty in the females. Pheromones in the wool, wax and urine of a ram are sufficient to stimulate ewes to ovulate, while the buck has a strong characteristic seasonal odor. The mere presence of the boar at the time of insemination of the sow improves sperm transport and ovulation, while the presence of the vasectomized bull has been reported to hasten the onset of puberty in heifers and also early resumption of ovarian activity in cattle following parturition [13].

2. Understanding pheromones and chemical signaling in bovines

2.1 Types of pheromones

On the basis of chemical structure, nature of molecules, mechanism of action, interactions and functions pheromones can be classified into various types, as described below:

2.1.1 Releaser pheromones

This type of pheromone elicit an immediate short term behavioral response (degraded quickly) either acting as attractant or repellent [14]. It has been observed that some organisms use powerful attractant molecules to get the attention of their mates as far as two miles or even more. These type of pheromones have been observed to stimulate immobilization reflex in sow, that is caused by the sex pheromones (released in the saliva) synthesized in the testes of boar, chemically identified as the steroids, 5α-androst-16-en-3-one and 5α-androst-16-en-3α-ol [13].

2.1.2 Primer pheromones

These pheromones mediate slow developing and longer-lasting changes to the endocrine state or development through activating the hypothalamic–pituitary–adrenal axis [13]. Priming pheromone resulted early puberty in prepubertal heifers receiving weekly oronasal treatments (7-week experimental period) with bull urine as against water-treated heifers [15]. Similarly, priming pheromones from males have been observed influencing induction of puberty, shortening of postpartum anestrus and the termination of seasonal anestrus in case of mammals, more pronounced effect observed in case of small ruminants [16]. Similar effect has been observed in case of gilts exposed to a mature boar, resulting in early onset of puberty and synchronizing effect on first oestrus [17].

2.1.3 Signal pheromones

These pheromones inform about individual or group identity crucial for parent-offspring recognition and mate choice. These pheromones cause short-term responses mediated by the central nervous system through neurotransmitter release, such as, gonado tropic releasing hormone (GnRH) in rats elicit a lordosis behavior. Teaser bulls exhibit flehmen behavior upon receiving the estrus specific chemical signal from females [13].

2.1.4 Major urinary proteins (MUPs) and odorant binding protein (OBPs)

First described in mouse and rat, MUPs and α2u proteins are lipocalins synthesized in the liver and excreted in the urine, and have got similarity with OBPs discovered in the nasal tissues of several vertebrates.

The MUPs have several important roles such as, transporting the pheromone in biological fluids, extending period of bioavailability by delaying the pheromone liberation, and modulating the pheromone activity [13].

2.2 Characterization of bovine pheromones

Dispersion of various estrus-specific compounds isolated from different samples such as vulvar swabs, vagina, urine, milk, feces, saliva and blood have been

demonstrated in the bovine body [18]. Nine estrus-specific compounds comprising of four amines, one ether, one alcohol, one diol, and two ketones, isolated from samples of estrus cows, tested positively in a bull behavioral assay [19]. Analysis of urine from cows at different stages of cyclicity had revealed presence of estrus specific pheromones such as 1-iodo undecane and di-n-propyl phthalate [20], the 1-iodo undecane have been also detected from feces of cows at different stages of the estrus along with acetic acid and proprionic acid [21]. Analysis of saliva have revealed five estrus-specific compounds, i-e, acetic acid, pentanoic acid, phenol 4-propyl, proprionic acid and trimethylamine, among which the role of trimethylamine in attracting the bull to the estrous cow has been proved in a bioassay [22]. Higher levels of 1-hexadecanol have been found in urine samples collected during estrous against the samples collected during the luteal phase, the compound supposed having a pheromonal effect in different animal species [23]. Various volatile compounds have been identified from bovine estrous urine such as 6-amino undecane, 2-butanone, coumarin, 1,2-dichloroethylene, 9-octadecenoic acid and squalene, found responsible for improving reproductive function of a bull in terms of enhanced libido and semen production [24]. Acetaldehyde an estrus specific pheromone from bovine vaginal secretions, was found helpful in predicting estrus and ovulation by monitoring its levels in blood, breath, milk, saliva, sweat, and its levels were found sharply decreased before, and at onset of estrus [25]. A gradual increase in the concentration of free fatty acids in estrous vaginal discharge before estrus and a sharp decline post estrus was observed, and it was noted that ruminal concentration of fatty acids affect the concentrations in urine but not in the vaginal discharge [26]. Quantification of methyl heptanol from vaginal secretion has been exploited as a method for detecting bovine estrus [27].

2.3 Biostimulation in bovines

Biostimulation (bull effect) a stimulatory effect of male upon female reproductive parameters, due to pheromones, result in the induction of oestrus and ovulation through genital stimulation, and therefore, has got potential in improving reproductive efficiency in livestock including bovines. It has been observed that significant proportion of heifers exposed to bull urine attained early puberty against the proportion of heifers not exposed, suggesting presence of priming pheromone in the bull urine [28]. Similar observation of early puberty attainment due to social interactions between bulls and prepubertal heifers [29], and also due to exposure to vasectomized bulls at significantly lower age of 23.1 months, as against 26.4 months in non-exposed heifers has been made [30]. In multiparous cows duration of post-partum anestrous was decreased when exposed to bulls [12], and interval to estrus was shorter in exposed cows [31]. Exposure to the androstenone a boar sex pheromone, was found having positive effect on reproductive parameters such as earlier onset of cyclicity at puberty and better results from artificial insemination in cows [32]. In a bioassay a mixture of compounds consisting of acetic acid, 1-iodo undecane and propionic acid from estrus cows were smeared on the genitalia of non-estrous cows, thereafter bulls were allowed to sniff the cows for 30 min, and it was observed that bulls displayed significantly longer flehmen behavior and increased number of mounts than the individual compounds and control, indicating the possible role of these chemicals in the induction of mating behavior [21]. Also various volatile compounds from bovine estrous urine (6-amino undecane, 2-butanone, coumarin, 1,2-dichloroethylene, 9-octadecenoic acid and squalene), resulted in improved reproductive function of a exposed bull in terms of enhanced libido and semen production [24]. Several mechanisms of biostimulation have been postulated such as, pre-estrus progesterone rise associated with bull exposure in

postpartum cows, resumption of cyclicity due to luteinizing hormone (LH) release following exposure resulting in positive feedback for LH release in the hypothalamus to estrogen (overriding inhibitory effects of low concentration of estrogen on the hypothalamus), and increased sensitivity of the ovary to LH by increasing the number of LH receptors [13, 31].

2.4 Application of pheromones in bovines

Pheromones have a lot of potential in modulating animal behavior, and enhancing cattle reproduction and management.

1. Pheromones have a role in attaining early puberty and decreasing duration of post-partum anestrous (discussed in Section 2.3)

2. Various pheromones have been exploited in diagnosis of estrus such as quantification of methyl heptanol from vaginal secretion as a method for detecting bovine estrus.

3. Pheromonetherapy, first used in the dog and cat, can be applied to animal species including bovines, in reducing anxiety and phobia, modulating animal behavior and helping to build a good maternal relationship. Pheromones have proved an economic and alternative role in estrus synchronization against hormonal treatment, early onset of puberty and reduction of postpartum anestrus period; and therefore, is a viable management tool in tropical areas, where livestock production is overwhelmed with constraints [33].

4. Pheromones have a role in influencing the standing posture and therefore, help in assisting artificial insemination, as has been observed in sows exposed to boar saliva, resulted in immobilization reflex [34].

5. The female urinary sex pheromones have a role in assisted reproductive technology by causing penis erection and increasing the amount of sperms in bovines [33].

6. The bovine appeasing substance (BAS) is a technology using appeasing pheromones consisting of a mixture of fatty acids, having composition similar to the original substance as produced by the dam at calving [35]. BAS has been used to reduce agonistic behavior in various animal species, thus stimulating feeding behavior and weight gain, and thus helped in improvement of post-weaning performance and reduced post-weaning mean haptoglobin concentrations. BAS administration helps in improved milk yield, reduced somatic cell count of dairy cows, and improved performance and reduced duration and costs of medication in case of pre-weaning dairy calves [35, 36].

3. Conclusion

Chemical messengers or pheromones have a direct developmental effect on hormone levels and behavior, and therefore, have a potential role in modulating animal behavior and reproduction management. The cattle pheromones acts as efficient means to decrease calving interval, post-partum anestrus period, optimized milk production, reducing anxiety and phobia, building a good maternal relationship, and helps in assisted reproductive, artificial insemination and BAS

technologies. Pheromones have proved an economic and alternative role in estrus synchronization against hormonal treatment, and their potential to transform the animal behavior and management of reproduction has led to development and use of synthetic pheromones to manipulate reproduction and behavior, and once chemically synthesized and characterized, have got huge scope of application that is beneficial for livestock owners.

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Chapter 16

The Incidence of Ovulation and Detection of Genes Associated with Ovulation and Twinning Rates in Livestock

Ozden Cobanoglu

Abstract

Cattle is a monotocous species that generally produce only one offspring per conception. However, multiple ovulations are a naturally emerging reproductive phenomenon typically controlled by genetic structure and environmental factors. On the other hand, few genes or causative mutations might explain significant genetic variations between animals for the reproductive traits. Studies report different methods, including QTL analysis, fine mapping, GWAS, and MAS selection, to improve such traits due to their economic importance. The recent fine-mapping study, which narrows the genomic region, indeed, influencing multiple ovulation, gives positive signals that causative mutation controlling high ovulation rate may be identified shortly. In conclusion, identifying the major genes that considerably affect ovulation and twinning rates provides the opportunity to increase reproduction efficiency by improving genetic gain in livestock species.

Keywords: ovulation rate, twinning rate, polymorphism, QTL, MAS, livestock

1. Introduction

Complex traits are typically influenced by multiple genes by their combined contributions and modifications of environmental factors. However, a few genes or loci account for most variation between individuals for any given domesticated species. Researchers develop various methods, such as marker-assisted selection (MAS), to improve production and reproduction, and performance traits because of their economic significance in dairy and beef cattle over the last 50 years. This chapter presents issues about the major traits with economic values for the genetic improvement of livestock reproduction. It also covers aspects from basic information about physiological mechanisms of ovarian follicular development in ruminants to incidence of multiple ovulations to the fundamental studies of ovulation rate in model species to all aspects of ovulation rates and genetic studies to identify quantitative trait loci or causative mutations affecting ovulation rates and more explicitly twinning rates in bovine species.

2. The overview of ovulation induction

Ovulation is the release of a fertilization-competent oocyte (mature female germ cells) from the ovary into the fallopian tubes in the female abdominal cavity where male sperm cells fertilize; thus, it is an essential and sophisticated biological process for sexual reproduction. Ovulation is an ovarian response that is initiated due to the surge of luteinizing hormone (LH) through the anterior pituitary gland. Thus LH surge triggers ovulation and estrus [1, 2] and the development of the corpus luteum, which initiates a series of ovarian activities in females. It works with the secretion of follicle-stimulating hormone (FSH), which plays a fundamental role in regulating for development of ovarian follicles as well as selection. It also stimulates granulosa cell differentiation, regulating gonadal functions, including steroidogenesis [3, 4]. LH, along with FSH, are considered gonadotrophic hormones because of their role in controlling the function of the ovaries in the development of preovulatory follicles to stimulate certain molecular events. This complex spectrum of events includes various types of ovarian cells, the activation of various signaling pathways, and the controlled expression of specific genes affecting the overall mechanism. LH and FSH levels are regulated and highly dependent on the pattern of release of gonadotropin-releasing hormone (GnRH) from the hypothalamus [5, 6].

Up to the last decade, a significant focus on ovulation and related features has been the association analysis of known candidate genes. The entire process of ovulation varies in mammals, following where they can be spontaneous or induced manners [7]. Spontaneous ovulation is the ovulation process in which females exhibit a constant cycle of reproductive hormones and does not require to be aroused in any way through a male to generate a preovulatory LH surge associated with reproduction. Species that are naturally ovulating through estrous produce mature ova through a process necessary for fertilization to occur. The females are ovulating spontaneously include mice, rats, domestic dogs, sheep, goats, horses, pigs, monkeys, and humans [8, 9]. The entire cycling process of ovulation varies among species. For example, while humans and primates experience monthly menstruation processes, all other animal species ovulate through various other ovulatory mechanisms [10].

Whereas female who displays mating-induced ovulation will have spontaneous development of follicles to maturation due to some component of coitus that is an externally-derived and receipt of genital stimulation during, or just before mating. Therefore, despite exhibiting high estradiol levels resulting from follicular maturation, they do not ovulate because they entail priming of males resulting in a long mating cycle to ensure successful fertilization [11].

Often, steroid-induced LH surges are not seen in ovulation types induced during reproductive periods, indicating insufficient or reduced secretion level of GnRH due to lack of positive feedback from estrogen and progestin hormones upon gonadotropin secretion. However, paradoxically, some natural ovulating species may undergo an occasionally induced preovulatory LH surge due to mating. Species in which females are triggered in induced ovulation include rabbits, domestic cats, ferrets, and camels [8].

Reproduction is a highly dynamic process and has significant consequences on livestock profitability. Reproductive success is conditioned by fertility, productivity, and fecundity. In particular, there are several minor genes and some major genes, which are fecundity related genes (Fec) that significantly affect reproductive traits, like ovulation rate, prolificacy, and litter size genetically [12].

Livestock species are mainly classified either as monotocous species, like cattle, water buffaloes, and horses, or multiparous species, like goat, sheep, and pig based on ovulation rate depending on the characteristic of a species [13].

Biological factors for the consistent multiple ovulations and how to improve or control the ovulation rate in other single-ovulating species in livestock have been of interest to some researchers to understand and intervene in the process of follicular development by applying assisted reproductive technologies. Therefore, identifying various experimental animal models with multiple ovulation rates could efficiently enhance the selection response in farm animals.

Specifically, the reproduction process is primarily influenced by genetic and environmental factors for a transformation from primordial follicle to mature ovulatory stage and typically has low to medium inheritance; thus, traditional phenotype-based selection methods are often time-consuming processes due to a lack of efficiency.

It is more effective to select breeding animals based on their genotypic structure to increase ovulation rate, prolificacy, and litter size as reproductive abilities in livestock species. Eventually, selecting animals based upon highly polymorphic marker information for reproduction efficiency (MAS) will be of great importance for future breeding programs in the livestock production system.

3. The use of molecular genetic markers and techniques to improve reproductive performance in livestock

Genetic improvement of reproductive efficiency is one of the most effective strategies available to improve the performance of farm animals. Especially in the last 50 years, selection program based on classical or molecular genetic principles has led to significant positive changes in dairy and beef cattle [14]. Reproductive efficiency is influenced mainly by environmental factors such as dietary regimen, animal health and management, and their interactions, as well as by many genes in dairy animals. Reproductive traits generally have low-to-moderate heritability and do not show excellent progression to phenotype-dependent selection by classic selection methods. Therefore, determining the genes that affect the reproductive ability and including them in the selection program is one of the crucial arguments in increasing the efficiency and success of the selection process.

Genetic markers of follicle number in cattle ovaries can identify heifers that will become highly fertile cows because genes play an active role not only in the physical structure of an organism but also in its functioning. Therefore, analysis of the farm animal genomes will enable us to identify putative genes that are supposed to affect fertility and cow productivity, which are economically important traits in livestock, as the ultimate goal. Specific chromosomal regions, which contribute to complex traits, are called quantitative trait loci (QTL). Several studies were conducted to identify genetic variation in quantitative traits in livestock and laboratory species since the genetic variation is an essential part of breeding programs. A possibility of detecting loci that affect quantitative traits using genetic markers has been realized since Sax's study with beans, which utilized seed-coat characters as markers due to the relationship with seed size in 1923 [15].

Selecting desirable alleles at particular loci based on marker information will increase the selection response for the next generation. Short sequences of DNA, called genetic markers, are specific DNA regions in the animal genome that indicate variation within the population. These polymorphic regions can be positively or

negatively associated with particular reproductive traits of interest. One of the main tools for genetic improvement is the wide usage of molecular markers such as microsatellites, minisatellites, and single nucleotide polymorphisms (SNPs) using different methods such as PCR-RFLP, SSCP, SSR, qRT-PCR, and whole-genome analysis or the next-generation sequencing [16].

Especially microsatellite markers are not only highly polymorphic but also reasonably abundant throughout the genome [17]. The relationship between marker alleles and phenotypic observations on the trait is used widely in linkage analysis to map a segregating QTL in a population. The presence of highly polymorphic DNA markers in genetic maps in various farm animals and their relationship to phenotypes provide an effective tool for QTL affecting traits. However, identifying markers closely linked to the target region and determining the association between marker allele and QTL allele, which control the quantitative traits, are rather complex processes. A high-resolution marker map and precisely recorded phenotypic values are needed to determine the linkage between marker loci and QTL with low to moderate effect controlling the traits like reproductive performance [18]. Therefore, the QTL region affecting mainly low-moderate heritable traits is detected to find molecular markers that can be applied in the MAS system, enhancing the genetic gain for the reproductive trait of interest.

Several reproductive traits have been associated with fertility in dairy cattle, including age at puberty, early ovulation, size of ovulatory follicles, multiple ovulation, ovarian cystic structure, embryo survival, and heat detection [19, 20], which heritability rates are around 1–5% [21]. The prediction of these heritability ratios still notifies that there is a potential to make genetic progress selecting against reproductive traits in bovine. Genome-wide association studies (GWAS) are widely used powerful techniques to discover genetic variants strongly associated with various complex traits concerning any disease resistance, productive and reproductive abilities in different organisms over the last twenty years. For this purpose, chip-based microarray technology has been developed as a high processing platform to support GWAS analysis. GWAS is a technique that assays high-density SNP markers located throughout the genome to identify putative locations, either causative or in linkage with continuous phenotypic variation. The availability of millions of SNPs markers makes the system easily genotyping on throughput platforms by covering the whole genome [22]. Various GWAS studies have been carried out on livestock, especially in dairy cattle [23], beef cattle [24], water buffalo [25], pigs [26], sheep [27], and goat breeds [28]. However, the large number of potential genes identified by GWAS have not been fully validated yet. As the best-powered studies, they are combining researches of GWAS data and genomic selection (GS) with MAS in livestock species will precisely accelerate the accomplishment of analyzing massive genotypic data through millions of genetic markers which are collected from up to hundreds of thousands of phenotyped animals with diseases and traits of interest soon [29, 30]. In addition, other new technologies, including RNA-sequencing technology, to be implemented through the genome-wide sequencing of mRNAs in animal species can be widely applied in such studies over time [31]. In conclusion, it is expected that many more major genes, causative mutations, and even several genes with minor effects will be definitively identified shortly due to the drastic decrease in prices for SNP genotyping and DNA and mRNA sequencing with the substantial increase in livestock genomic studies.

4. Developmental stages of ovarian follicles

Folliculogenesis, the complex biological process of forming ovulatory follicles among the cohort of growing primordial follicles on the ovaries produced by

female animals throughout their lifetimes, causes changes in ovarian morphological characteristics during the typical estrus cycle, an essential aspect of female reproduction [32]. Cattle are a monovular species that can produce several hundred thousand primordial follicles at the onset of puberty, depending on their physiological mechanism. However, practically less than 1% of these follicles will grow and be ovulatory in the late stages of development due to atresia. Selecting a single dominant follicle among many growing primordial follicles is an essential step in livestock reproductive technology. Therefore, any intervention or malfunction in this process can lead to infertility or multiple ovulation in females. The current follicle selection process focuses on the role of follicle growth and selection of the dominant follicle regulated by LH, FSH, and insulin-like growth factor family (IGF) hormone mechanisms [33].

Many studies on the growth stages and developmental processes of follicles in animals have also presented different models. As one of the most notable models, Rajakoski proposed the developmental stages of antral ovarian follicular growth in cattle occur in a wave-like pattern [34]. Many researchers reported that each cycle usually involves two or three waves. As a result of applying transrectal ultrasonography technology, the concept of follicular waves known to that day has been re-investigated and facilitated the understanding of the pattern of follicular waves during the estrus cycle [35, 36]. Therefore, ultrasonography technology has provided more detailed information about the follicular developmental stage and the follicular wave dynamics. In addition, monitoring the growth and development patterns of follicles has enabled us to make more detailed observations about follicle selection and understand how it relates to the endocrine secretion mechanism during the maturation of follicles [36, 37].

First, Pierson and Ginther observed individual follicular development during the cycle using this technology [37]. Later, various intensive studies were carried out to investigate these developmental stages of animal husbandry, especially in sheep and cattle breeds [38, 39]. The follicular wave pattern in ruminants is typically two or three follicular waves per cycle in cattle; incredibly primitive and very fertile dairy cows usually have two wave cycles, while nulliparous dairy heifers aged 2–2.5 years have three-wave cycles [40]. However, it is three to six waves per cycle in sheep [41]. Studies were proving that the developmental processes of follicles within a follicular wave are highly variable among waves. After puberty, all primordial follicles have an equal chance of becoming mature follicles. Primary follicular wave is characterized as the synchronous growth of a group of small antral follicles. One of them is eventually selected to be dominant and thereby becoming ovulatory among the group of follicles within each follicular wave. But all other remaining "subordinate" follicles of the ovulation wave will regress and degenerate during the typical estrus cycle [42, 43]. The dominant or mature follicle of the wave is typically the largest in diameter. Still, the subordinate follicles belong to the same group of follicles which the dominant follicle comes from [40].

Traditionally, in cattle, the day when a follicular wave can be first detected determines the day when the first observation of the dominant follicle can be made retrospectively [44]. A first dominant follicular wave emerges when the follicles are 4–5 mm in diameter at approximately the day of ovulation [42]. Subsequently, a second ovulatory wave can be detected about 9 or 10 days later [45]. The main event that causes single ovulation to occur in cattle is called follicle selection. Diameter deviation occurs approximately 2 to 3 days after the emergence of the follicular wave in the selection of follicles in the morphological process. Thus, while the future dominant follicle grows continuously, the growth rate of the lower follicles slows down, and then their growth is stopped entirely, and they undergo degeneration. Although this deviation varies among individual animals, it is widely accepted

as it has been observed in this range in many studies using both Bos taurus and Bos indicus breeds [46–48]. However, the high progesterone concentration prevents the first dominant follicle from maturing, as the corpus luteum has not regressed yet. Thus, the first dominant follicle cannot be functional and ovulatory. Subsequently, a second ovulatory wave can be observed. The dominant follicle from this wave can keep on growing and ovulating during the corpora lutea (CL) regression. In addition, a third source of the ovulatory follicle becomes apparent on day 16 after ovulation in some cattle breeds due to the regression of the second dominant follicle during luteolysis. Even if each wave involves simultaneous emergence of a cohort of follicles, usually one of them, sometimes two, become dominant follicle(s), and all of the others eventually become subordinates. A single oocyte is released from the dominant follicle due to either naturally occurring or artificially induced ovulation. On the other hand, subordinate follicles begin to regress right after a short growing phase [44, 45].

It was noticed from individual to individual that the follicle size at ovulation was quite different. Dairy heifers showing two-wave cycles have a follicle at a diameter of 16.5 mm in ovulation. However, follicle size is smaller in heifers (13.9 mm) with three-wave cycles [44]. Similarly, the size of ovulation follicles has been reported as 14.8 mm in Holstein heifers. However, the follicle size observed for lactating dairy cows was slightly larger and was found to be 17.4 mm [49]. In many studies of follicular diameter deviation, both the future dominant follicle and the most significant lower follicle were more prominent in Bos taurus. However, diameter deviation occurred at similar times after wave emergence in Bos taurus and Bos indicus. Bos indicus has a smaller follicle size when the deviation in the follicle diameter cannot be fully revealed. Nevertheless, the results of the studies support the idea that the future dominant follicle generally has a size advantage over the largest subordinate one [46, 48, 50].

Reproductive biotechnology has recently emerged as a powerful tool to improve livestock productivity and reproductive performance. Therefore, these modern reproductive technologies have started to be used instead of conventional classical techniques in many reproductive-based studies recently. Progress in our understanding of follicle development and selection has sparked the development of synchronization protocols for fixed-time artificial insemination (AI), in addition to the applications of other cutting-edge reproductive technologies such as in-vitro fertilization (IVF), embryo culturing and transfer (ET), cloning, estrus synchronization, transgenesis, and much more new emerging reproductive biotechnologies [51, 52]. As a result, these developments in terms of sustainable livestock productivity are important for optimal follicle growth and making the right choices to increase reproductive efficiency in livestock species.

5. The incidence of multiple ovulations

Cattle are a uniparous species that means females usually produce only one progeny per conception due to the single dominant follicle in each ovulatory cycle. Alterations in follicle selection can lead to codominant follicles and multiple ovulations, which are the basis for multiple births in cattle and sheep [53]. In rare cases, the synchronous emergence of two follicles as a physiological pattern, albeit in a monovular type, is altered so that the follicle selection mechanism allows both to be selected as the dominant follicle among several follicles in the follicular wave. The ease of evaluating follicular events by trans-rectal ultrasonography and the accuracy of the data obtained from these studies have allowed cows to be widely used as an ideal research model in follicular studies in ruminants and humans [54, 55].

Ultimately two oocytes are released from codominant follicles at the end of ovulation due to either natural stimulation or artificial inducements. In the development of codominant follicles, deviations occur in the diameter of the follicles when the largest follicle and the second-largest follicle are close to 8.5 mm. The third-largest follicle has a low growth performance, and the deviation in 2nd largest follicle may occur 36–50 h after the deviation of the first follicle [56]. Ovulation of two future dominant follicles occurs either from the same ovary simultaneously, or each follicle consists of a separate ovary [57]. In a study, synchronous production of two oocytes from different follicles was observed due to the evidence of two corpus luteum (CL) on the ovaries of cattle [58]. Also, research about follicular development during the estrous cycle in twin-calving cows indicated that double and triple ovulations coincide from different ovulatory follicles of the same follicular wave rather than ovulation of single mature follicles from two consecutive waves [59]. In addition, the authors noted that the cysts in the ovary and lack of CL possibly increased the incidence of double ovulation during pregnancy [60]. Therefore, as more than one follicle deviates and becomes dominant, the chance will be increased for ovulation of more follicles simultaneously. After all, twins, triplets, or overall multiple births in rare circumstances will become a reality if all subsequent events commonly occur for both oocytes from fertilization to parturition.

The natural incidence of twin or triplets birth in cattle is mainly due to multiple ovulations that have been summarized in many studies, even if the results are inconsistent [61]. While the multiple births five decades ago is around 1–5% depending on breed, genetics, parity, and other environmental conditions [62], this rate has increased up to 10–22% in lactating dairy cows today. There have been many studies conducted about regulating multiple birth rates in cattle by selecting genetically favorable animals [63], utilizing hormonal treatments [64], or utilizing embryo transfer techniques [65]. One of the reasons affecting ovulation rates is low progesterone secretion in older cows, which might be the main reason for the increase in circulating LH level and eventually causes enhance ovulation numbers as progesterone has a suppressive effect on LH release. In addition, growth hormone and nutritional treatments greatly influence a multi-ovulation response of an individual [66]. Also, the ovulations of two follicles simultaneously caused to increase in days of milk among pregnant animals [60]. In a recent study, the incidence of multiple ovulation rates in early lactating animals was 14.1%, but they did not significantly affect various reproductive outcomes of cattle [67]. Although the underlying mechanisms of multiple ovulation have been studied extensively, the dynamics of the entire mechanism are still not fully explained.

Monozygotic twins are genetically and physically identical since they are formed from one fertilized egg, splitting into two identical halves during early embryonic developmental stages. Thereby both individuals are always the same sex. In the case of dizygotic or fraternal twins, two different sperm fertilize two completely different ova simultaneously. Thus the successful result of ovulation and fertilization of two oocytes will be dizygotic twins. Dizygotic twins are not identical genetically or phenotypically as monozygotic twins are. They are not necessarily the same sex as opposed to monozygotic twins. They can also be similar or different from siblings born from the same parents during different gestations [68].

Twin or triplets birth is an unavoidable issue in dairy and beef cattle production systems which negatively affects the health, production, reproduction and overall decreases the productive life span of animals [69]. The study reports that the calf survival incidence from twin- and triplet-producing animals were relatively low, around 44% depending on the breed composition [70]. In a recent study of the economic analysis of multiple births, the economic loss to the livestock breeder from each twin calving was estimated at between $59 and $161 in cow-calf

production systems [71], even if twin calving could reduce substantially beef meat production costs owing to more calf growth at weaning [69]. Thus twin or triplet calving causes to lessen overall cow reproductive efficiency, productivity, and thus the profitability of enterprises. In conclusion, a complete understanding of the complex process of follicular growth during the estrus cycle and the development of oocytes will undoubtedly improve the knowledge to maximize and control the efficiency of reproduction in livestock species, especially the existence of dizygotic twinning since the fertilization of more than one oocyte after ovulation will be the main reason of multiple births.

6. Studies on ovulation rate in small ruminants as a model organism

Detection of the specific major genes that control reproduction traits provides the opportunity to improve genetic gain in livestock species. However, fertility traits generally have low heritability, and reproductive improvements in a phenotypic selection based on observable data are pretty low and limited. However, ovulation rate and litter size in sheep are important fertility traits, and they have high economic values for breeders [72]. The ovulation rate mainly determines the productivity of sheep. Ovulation rate and litter size are expressed in only one gender and can only be recorded relatively late in the animal's lifespan. Focusing on improving fertility traits will have a long-term impact on the profitability of the sheep production system [73]. For more than a decade, sheep have been used by many researchers as an essential model organism to identify genes that control reproductive functions such as high fertility rate and ovarian follicle selection and also to investigate the physiological mechanisms involved in this reproductive system. Strong evidence was detected for major genes controlling prolificacy in sheep [74]. Specifically, the genetic influence on prolificacy variability in sheep has demonstrated that many genetic mutations have essential roles in controlling the ovulation rate. Those genes were tested in several populations based on the patterns of phenotypic segregation. Therefore, the selection of breeding animals will be more effective based on genotyping for relevant candidate genes to improve fertility and fecundity traits such as ovulation rate and litter size in small ruminants.

In a study conducted in this context, it was observed that sheep developed from the Booroola Merino strain had an autosomal mutation that increased the ovulation rate by approximately one and a half ova [75]. Therefore, it was an excellent candidate to investigate the mechanisms controlling ovulation rate in mammals [74, 76]. It was reported that the Booroola fecundity gene (FecB) has a partial dominant effect on litter size due to embryonic loss in homozygous carrier animals with high ovulation rates [77]. Subsequently, it was observed that, on average, one copy of the FecB gene enhanced the number of offspring by about 1.5, with increasing ovulation rates of about 1.65 ova per copy of the gene. The gene was accurately mapped to chromosome (Chr) 6 in a region where the bone morphogenetic protein receptor 1B (BMPR1B) was located in sheep [78]. This region is syntenic to Chr 3 or 5 in mice and Chr 4 in humans [79].

Moreover, the other major gene was detected, increasing ovulation rate and litter size in Inverdale sheep. The Inverdale fecundity gene (FecXI) has been located on the X chromosome and increases ovulation rates in the heterozygous ewes [80]. But, homozygous ewes are observed to be infertile due to lack of follicle development [81]. Another fecundity gene (FecXH) was also identified successfully on the X chromosome in the Hanna sheep population [82]. Both FecXI and FecXH were mapped in the bone morphogenetic protein 15 (BMP15) site. However, different point mutations were identified in the BMP15 gene in Inverdale and Hanna sheep

populations. If ewes are heterozygotes for any of them, it causes to increase ovulation rate to two-three ova. However, if sheep is homozygous for Booroola mutation, it dramatically raises ovulation rate from 5 to 14 [83]. Another study investigating the ovulation rate records obtained from daughters of ewes inseminated by Coopworth rams to understand the inheritance pattern of ovulation rate also proved that there was another maternally inherited gene affecting productivity traits located on the X chromosome (FecX2w). But the location of this gene is entirely different from the gene on Inverdale FecX locus [80]. The findings of studies conducted about four decades ago have guided many subsequent types of research on this subject, in which sheep are extensively used as model organisms in this subject.

Currently, the segregation of five major genes that affect ovulation rate and prolificacy has been characterized at the molecular level in various sheep and goat breeds that cause significant phenotypic variations. Overall the detected genes are bone morphogenetic protein receptor, type 1B (BMPR1B; in Booroola Merino, Javanese, Small Tail Han, Hu, Garole, and Kendrapada breeds) [78, 84, 85], bone morphogenetic protein 15 (BMP15; in Inverdale, Hanna, Romney, Belclare, Cambridge, Galway, Lacaune, Raza Aragonesa, Olkuska, and Grivette breeds) [82], growth differentiation factor 9 (GDF9; in Belclare, Cambridge, Icelandic Thoka, Santa Ines, Embrapa, Finnish Landrace, Norwegian White Sheep, Ile de France, and Baluchi breeds) [86], beta-1,4-N-acetyl-galactosaminyl transferase 2 (B4GALNT2 in Lacaune) [87], and leptin receptor (LEPR in Davisdale sheep) [88]. Causative polymorphism studies in different prolific sheep breeds showed at least 12 identified allelic variants for the BMP15, BMPR1B, and GDF9 genes encompassed in the transforming growth factor-beta (TGF-β) signaling pathway secreted from the oocyte. TGF-β is significantly associated with ovulation rate, litter size, and prolificacy and thus plays a critical role in the folliculogenesis of small ruminants. Many studies reported that the mutations in TGF-β pathway-related genes enhanced ovulation rate (35–100%) in heterozygous animals [89]. Moreover, even if causative mutations for fecundity are not fully discovered, two other genetic variants were identified as FecX2W [90] and FecD [91], which are segregated in prolific sheep breeds in recent studies. Similarly, about 20 different candidate genes, including TGF-β related genes, were also detected to play a crucial role in regulating folliculogenesis and prolificacy-related traits in several goat breeds [28]. To improve the genetic makeup of animals affecting high productivity in livestock, over 30 small ruminants, mostly high-yielding sheep and few goat breeds, have been actively used in candidate gene studies that focused on detecting variation related to reproductive performance-related traits.

7. Ovulation rate studies in bovine

7.1 Cattle as a model animal for multiple ovulation

As a uniparous species, cattle produce only one progeny in most cases, resulting from ovulation of a single follicle during the pregnancy. Nevertheless, the natural incidence of twin or triplet calving in cattle is mainly due to multiple follicular ovulations concerning breed differences, age of dam, parity, season, the effects of feeding and management systems, geographic location of raised animals, and other environmental effects [62]. Specifically, the incidence of double birth was observed as approximately 1% in beef cattle [92]. In comparison, this rate was determined as 4–5% on average, ranging from about 1% for heifers to nearly 10% for older cows in dairy cattle [93, 94]. Several studies were conducted concerning underlying causes of multiple ovulation rates, particularly twinning rates in cattle by selecting

genetically highly polymorphic animals [63], using trans-rectal ultrasonography or quantifying by circulating AMH concentrations, utilizing embryo transfer techniques [65], or utilizing hormonal treatments [64].

The ovulation rate is closely related to the twinning rate in cattle due to the high genetic correlation between ovulation and twinning rates, ranging from 0.75 to 1.0 [95]. Although the genetic control of multiple ovulation in cattle by major genes has long been the subject of research, and there has been significant interest in the mechanism underlying multiple ovulation in bovine species [70], genes with significant effects on ovulation rate have unfortunately not been identified until recently [96].

7.2 QTL studies about twinning and ovulation rates

The selection of genetically superior animals in terms of twinning frequency has been practiced in long-term experimental herds in different countries. For this purpose, various research herds for multiple ovulation studies have been implemented to be established in various countries for four decades. These herds were begun to set up in the early eighties to select for increased twinning rates in France [97], Australia [76], New Zealand [98], and Meat Animal Research Center (MARC) of the USDA-ARS in the USA [99] to develop effective genetic strategies to improve production efficiency including twinning and ovulation rates, meat quality, and animal health in dairy and beef cattle production. MARC twinning population initiated with a total of 307 well-suited cows from twelve different experimental beef, dairy, and dual purposes breeds to study involved in follicular development and recruitments and identify genes affecting primarily twinning rate; later taken into account of ovulation rate in 1981 [63, 100]. These cows were selected based on their high twinning frequencies. The twinning rate can be defined as sequential events due to ovulation, conception, and embryonic survival [101]. Sires whose dams were founders of the herd and sires whose daughters had high twinning rates were used for breeding the founder cows. In addition, semen collected from sires that mainly originated from Swedish and Norwegian breeds was used in the project. The founder breeds in the herd were mainly Holstein (18%), Swedish Red and White and Norwegian Red (12.8%), Swedish Friesian (16.1%), Pinzgaurer (18.4%), Simmental (15.8%), Charolais (5.3%), Angus and Hereford (8.3%), and other breed crosses (5.3%) [102]. The primary objective of the research was to increase the twinning rate in the herd. Therefore, they selected animals based on twinning performance. However, later on, they also evaluated animals' ovulation rate records for 8 to 10 estrous cycles since ovulation rate is highly genetically correlated with the twinning rate (0.90) [58]. Thus, they used an animal model with multi-trait repeated records to predict breeding values for twinning rates in 1990. By applying this methodology, they were able to use information not only from the individual but also from all available relatives for twinning and ovulation rates. The most significant advantage of using ovulation rate records as an estimator of twinning rate is to reduce generation interval and reduce the number of cows retained for several generations. The estimated twinning rate was about 4% in 1984. But this prediction rose linearly to 35% in 1996 [100]. In the latest report, all the cows with lower estimated breeding values (EBV) were culled from the herd. Thus herd size was reduced from 750 to 250 cows giving birth annually. The twinning rate then was enhanced from 35% to over 50% annually since 1997 [103, 104].

Many studies have been conducted to identify ovulation rate and twinning rate QTL in different cattle populations. Several genomic regions for putative ovulation rate were detected on BTA7 and 23 [105], on BTA5, 7, and 19 [106], on BTA5 [107, 108], on BTA7, 10, and 19 [109], on BTA14 [101] for ovulation rate using the USDA

Meat Animal Research Center (MARC) twinning herd, a herd with a substantial contribution from Holstein–Friesian and Norwegian Red breeds [110]. A suggestive twinning rate QTL on BTA5, 7, 12, and 23 have been identified in the Norwegian dairy cattle population [111, 112]. Twinning rate QTL based on genome-wide searches have also been observed on BTA5, 7, 19, and 23 [113], on BTA8, 10, 14, 21, and 29 [114], on BTA2, 5, and 14 [115] in North American commercial dairy cattle populations; on BTA6, 7, and 23 in the Israeli-Holstein cattle using daughter design [116], on BTA20 and 28 in the INRA experimental herd selected for twinning [117]. In studies using composite MARC herds, it was determined that cows producing twins based on genetic selection for high twinning and ovulation rates over multiple generations produced about two-fold more secondary follicles than animals in the control groups. The probable reason for the higher twinning and ovulation rates in this herd may be the combined effects of multiple genes associated with these quantitative traits [63, 100]. Multiple positional candidate gene regions associated with ovulation rate, twinning rate, and multiple birth rates in various cattle breeds have been identified by linkage analysis, interval mapping, linkage disequilibrium (LD) analysis, the combined linkage-linkage disequilibrium analysis (LDLA), and GWAS analyses even if only a few have been replicated. Depending on the statistically significant level, the QTL or single nucleotide polymorphisms (SNPs) determined in the studies so far were diverse throughout the bovine genome. They spanned about 18 of the 30 bovine chromosomes given in **Table 1** [89].

It is noteworthy that a crucial QTL region was detected on BTA5 in the MARC experimental herd, commercial dairy cattle populations raised in North American and Norway. Some of the founder sires in the composite MARC population were originated from Scandinavian countries whose progenies gave multiple births. Therefore, the probability of detecting the same QTLs in future studies is quite high due to sharing a significant portion of the founder genes in two different populations [63]. Furthermore, different studies have reported that IGF-1 as a candidate gene (especially the 2nd intronic region) in BTA5 is substantially associated with the twinning rate in US Holstein cattle [118, 119]. The presence of several QTLs for twinning rate and ovulation rate was detected, which were spanned 24 out of the 30 bovine chromosomes as a result of studies using high-throughput single nucleotide polymorphism (SNP) genotyping throughout the genome based on linkage (LE) and linkage disequilibrium (LD) analyses.

7.3 Novel candidate genes affecting multiple births in cattle

In cattle, twin or triplet births are naturally occurring reproductive processes, although not a joint physiological event in bovine. Models derived from the study of high prolific sheep breeds provide a framework for searching the regulation of follicular development in monotocous species, such as in cattle or humans.

A highly fertile cow named 'Treble' was born in 1993 at one of the cattle herds in New Zealand. Although the breed's origin is unknown, it has been assumed to likely include a hybrid of Hereford, Holstein, Angus, and Jersey breeds based on the coat color pattern. Treble calved three sets of triplets her life span as one heifer and two stillborn calves at the first time in 1995, two heifers and one bull, named as Trio at the second time in 1996, all stillborn calves due to considerable difficulty during the delivery period at the third time in 1999. Treble was cloned later, and two clone progenies were born in AgResearch Centre, NZ, in 2000. On the other hand, a son of a highly prolific cow named the 'Triple' was bred with a group of cows with high calving rates that had several progenies by 2008. Thirteen daughters out of his total of forty-four daughters produced a total of fifteen twin and six triplet sets, where triplet calving were 29% of all multiple calving, supporting the idea of a naturally-occurring

Trait	Chr (Appx. location as cM)	Population	Positional candidate genes (Chr)	Method	References
Ovulation rate	7 (40), 23 (27)	MARC Twinner	*CYP21* (23)	Interval Map.	[105]
Ovulation rate	5 (107), 7(5, 57) 19 (65)	MARC Twinner		Interval Map.	[106]
Ovulation rate	5 (40)	MARC Twinner		Interval Map, Assoc./LA	[107, 108]
Ovulation rate	7 (30), 10 (75), 19 (65)	MARC Twinner	*AMH* (7), *ESR2* (10), *IGFBP4* (19)	Interval Map.	[109]
Ovulation rate	14 (61)	MARC Twinner		Interval Map.	[101]
Twinning rate	5 (68), 7 (108), 12 (9), 23 (30)	Norwegian Cattle	*IGF1* (5), *CYP21* (23)	Interval Map.	[111]
Twinning rate	5 (64)	Norwegian Cattle	*MGF*	LDLA	[112]
Twinning rate	5 (68)	US Holstein	*IGF1* (5)	Interval Map./ LDLA	[113, 115]
Twinning rate	8 (117), 10 (41), 14 (68)	US Holstein		Interval Map.	[114]
Twinning rate	6 (55), 7 (25), 23 (67)	Israel Holstein	*AMH* (7), *CYP21* (23)	GWAS	[116]
Twinning rate	10 (49), 20 (27), 28 (8)	INRA Twin		LDLA	[117]
Twinning rate	4 (44), 5 (67), 6 (8,44), 7 (68,76), 8 (58), 9 (34), 11 (47), 14 (21, 38), 15 (23), 23 (51), 28 (9)	US Holstein	*IGF1* (5)	LDLA/GWAS	[118, 119]
Twinning rate	10 (14)	MARC Twinner	*SMAD3*, *SMAD6*, *IQCH* (10)	Linkage/Fine Map.	[120]
Twinning rate	24 (40)	Italian Maremmana	*ARHGAP8*, *TMEM200C* (24)	GWAS	[121]
Multiple birth rate	11 (31)	Swiss Holstein and Simmental Cattle	*LHCGR*, *FSHR* (11)	GWAS	[122]

Table 1.
Chromosomal locations of quantitative trait loci (QTL) and single nucleotide polymorphisms (SNP) associated with ovulation rate, twinning rate, and multiple birth rate in various cattle breeds [89].

major bovine allele contributing to a high fecundity rate in a family of cattle with triplet calving ability throughout the generations in New Zealand. The possible scenario for this situation might be that a gene or set of genes should be segregated as a single copy from a dam (Treble) to some descendants through its son (Trio) for single gene inheritance. Moreover, such a unique gene allele is expected to be segregated as dominant or partially dominant in female animals [70].

Several daughters (131) of Trio were born by AI in the USA by following the importation of his sperms at a University of Wisconsin (UW)-Madison research farm from 2008 to 2011. The research reports that a significant bovine allele for high ovulation was identified and mapped on a 2-Mb window on BTA10 (+1.02 CL per cycle for carriers vs. noncarriers for the marker allele of the high ovulation rate) by using fine mapping techniques employed the animals raised at UW-Madison research farm [120]. Thus, the daughters of Trio proved that there was evidence of a high-fecundity allele transmitting on BTA10 that had a major influence on multiple ovulations in cattle [96]. The detected location was not overlapped with any major genes previously reported for the high ovulation rate and litter size in prolific sheep breeds. Eventually, in addition to the noteworthy reproductive performance of Treble, all of her descendants, including Trio, also displayed extraordinary reproductive performance. Therefore, the members of the Treble family with highly reproductive ability should be heavily employed in gene mapping studies to discover major genes with high fecundity rates. It can provide a significant resource for the subsequent investigation of genetic diversity in bovine productivity [70]. In the follow-up study, the location of a major gene for high ovulation rate was strongly detected at 1.2 Mb region of BTA10 using half-sib daughters sired by a bull that assumed to be carriers of the Trio allele due to a single mutation. It is noteworthy that the novel region obtained does not overlap with any major gene previously reported, which significantly affecting ovulation rates in ruminants. Thus, the study reports that the newly identified regions could be employed to track inheritance patterns for multiple ovulation rates using from the carrier father's lineage since the screening of the aforementioned candidate gene consist of any functionally putative causative mutations in the coding region and 5′ or 3′ flanking regions, reminding that the polymorphic SNP region might affect the expression level of any candidate gene controlling the high reproductive performance of animals [96]. When the follicular and hormonal dynamics of animals carrying the high prolific Trio alleles were examined in animals raised at UW, the Trio carrier animals displayed multiple ovulation. The carriers produced more dominant and ovulating follicles with smaller diameters and volumes in this process due to the slower follicle growth rate close to the beginning of deviation during the entire follicular wave. In the study, even if the deviation times were similar between heterozygous bearing allele from Trio and half-sibling noncarriers, a significant increase in the selected number of multiple dominant ovulatory follicles in cow having Trio allele was reported to be associated with the enhanced concentration of FSH secretion close to the deviation time in the follicle. There was also evidence that smaller-sized follicles had more LH receptors in animals carrying the Trio allele than noncarriers, supporting the potential novel physiological mechanisms causing the production of multiple ovulatory follicles in the Trio allele carriers [89].

This newly identified candidate region covering 1.2 Mb in BTA10 contains seven protein-coding genes, of which three of them might be taken into account as putative candidate genes. These genes are the small-mothers against decapentaplegic (SMAD) family member 3 (SMAD3), SMAD family member 6 (SMAD6), those of which are the primary signal transducers for the receptors of the transforming growth factor-β (TGFβ)/Bone Morphogenic Protein (BMP) superfamily ligands [123], and IQ motif containing H (IQCH), which is strongly related with the first menstrual cycle in human females [124]. The other follow study stated well-conserved SMAD6 gene, which plays a crucial role in preventing the BMP/SMAD-dependent signaling pathway, was 9.3 times more expressed in carrier animals for the high fecundity Trio allele versus noncarriers using animals in UW-Madison research farm by applying quantitative real-time PCR technique.

Ultimately, the effect of over-expression of the SMAD6 gene displayed a similar impact of causative mutations on the functions of BMP15, BMPR1B, and GDF9 genes as part of a signaling pathway that may alter the incidence of ovulation rate upward in prolific sheep breeds [125].

In another study to determine the genetic basis of the observed increases in twinning and calf mortality in Italian indigenous Maremmana cattle breed, the most significant SNP markers (Hapmap22923-BTA-129564) were located near two genes, ARHGAP8 and TMEM200C on BTA24, which could be putative functional candidates for cattle twinning rates [121]. Furthermore, in a very recent study, the researchers detected a major QTL mapped to a 70 kb window between 31.00 and 31.07 Mb on BTA11 for multiple maternal births, explaining approximately 16% of the total genetic variation based upon linkage-disequilibrium analysis (LD) using the whole-genome sequence information of the Swiss cattle population. The identified QTL includes the LHCGR and FSHR genes as functional candidate genes. Precisely, a regulatory variant in the 5′ non-coding region of LHCGR is predicted as a potential causative mutation for the QTL region [122].

8. Conclusions

These studies covering the physiological mechanisms regulating ovarian follicular development through multiple births displayed us the reproductive traits are highly complex traits that involve a potential genetic background and significant contributions of various environmental factors. Several studies report that causative mutations in TGF-β pathway-related genes, including BMP15, BMPR1B, and GDF9, strongly affect the ovulation rates in prolific small ruminants, which tend to conceive and maintain multiple ovulation spontaneously. Even if the bovine is naturally low-ovulating mammals, some variations may still be observed in the ovulation rates, despite the low heritability; thus, there is a potential to make genetic progress through selection against reproductive traits by using multiple observations of ovulation rate as the indirect selection criterion in cattle. Recent fine-mapping studies that narrow the genomic region truly, influencing multiple ovulation, especially on BTA5, 10, 11, 14, and 24, give positive signals that causative mutation controlling high ovulation may be identified shortly.

The complete understanding of the complex process of follicular growth during the estrus cycle and the development of oocytes will undoubtedly improve the knowledge to maximize and control the efficiency of reproduction in livestock species, especially the existence of twin or triplet births since the fertilization of more than one oocyte after ovulation will be the main reason for multiple births. On the other hand, it should not be ignored that many factors, including genetic but primarily environmental sources, specifically breed differences, age and parity of dams, the season of calving, and the effect of feeding and management systems significantly affect ovulation and twinning rates importantly in especially cattle production systems. Therefore, the production of twin calves might be more profitable for the breeder by implementing appropriate management and feeding programs to cope with the reproductive problems faced by twin-bearing cows.

In conclusion, understanding the genetic background of high fertility in mammals, on the one hand, is extremely important for the designing of convenient genetic improvement and management programs in livestock; on the other hand, it provides the basic knowledge necessary to overcome fertility problems in humans by using the cow as the ideal model organism.

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Chapter 17

Future of Bovine Amniotic Membrane: Bovine Membrane Application on Wound Healing, Surgery and Prospect of Use for Urethral Reconstruction

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Abstract

This chapter describes how bovine amniotic membrane could be indicated for wound healing, especially in complex surgery such as urethral reconstruction. Chemical studies have assessed both histologically and immunohistochemically that bovine amniotic membrane creates scaffold for wound healing. Whereas, clinical studies have shown that bovine amniotic membrane property could be substituted for wound dressing hence improving skin or mucosal integrity. Bovine membrane has been known to be used for many specialties such as ocular surgery, neurosurgery, maxillofacial and orthopedic surgery. This chapter includes such studies and shows the usage possibility of bovine amniotic membrane for other complex defect as shown in urethral reconstruction.

Keywords: bovine amniotic membrane, scaffold, urethral reconstruction, wound healing

1. Introduction

Fetal development in mammals is a complex pathway which occurs after prenatal embryonic development [1]. Fetal development system is achieved by meticulous interactions consisting in umbilical cord, amniotic fluid and placenta. Fetal membrane is part of the system, composed of two layers: an outer layer (chorion), which contacts maternal cells and an inner layer (amniotic membrane; AM). Fetal membrane holds important role in fetal development, essential for protection, breathing, nutrition and excretion. Amniotic membrane or amnion is a thin membrane on the inner side of the placenta forming a sac that completely surrounds the embryo/fetus and delimits the amniotic cavity, which contains amniotic fluid [2, 3]. AM holds important metabolic roles by transporting water, other soluble materials and the production of bio-active factors, including vasoactive peptides, growth factors and cytokines. AM also provides the fetus with protection against dessication and environment of suspension, thus promoting embryonal growth. This function is mainly attributed by tractional resistance

mainly related to the condensed layer of interstitial collagen type I, II and elastin [2, 4]. Toda et al. mentions that amnion also holds pluripotent differentiation ability with low immunogenicity and anti-inflammation property [5]. These properties creates opportunities and interest on the use of amniotic membrane for regenerative medicine.

The usage for human amnion as a surgical material for skin substitute was first suggested and reported by Davis in 1910. Sabella in 1913 performed the first human trial for skin grafting [6–8]. Bovine amnion first use for absorbable insulating material and suture material was described by Johnson in 1937. Bovine amniotic membrane (bAM) and bovine allantoic membrane was suggested as biological dressing [9]. A study by Rao stated that further preliminary experimental study using bovine amnion xenograft was suggested by Silvetti et al. in 1957 [6]. Since then, there are many basic and clinical researches regarding usage of bovine amnion on regenerative medicine.

Bovine amnion was worth noting, due to its wide surface area compared with human amnion (6000–7500 sq. cm vs. 1600 sq. cm) and similar histological appearance. Both amnion characteristic is marked with single layer of cuboid epithelium [6]. Consideration should be taken for the AM harvest timing due to morphological change during gestational period. Morphological change of bovine amniotic membrane has been observed between 40 and 230 days of gestation. The epithelium changes from a single layer of flattened, squamous cell containing conspicuous cytoplasmic organelles into single or multi-layered cuboidal cells with numerous microvilli. This epithelium is further supported by a basement membrane rich in collagen. The extracellular matrix is infiltrated with fibroblasts, mesenchymal stromal cells, and tissue macrophages ("Hoffbauer cells") [10].

Proteomic profile of bovine amniotic membrane is proved rich in proteins and signaling pathways. An analysis study by da Silva in 2021 identified 2105 proteins with an interactive network of 1271 nodes (proteins) and 8757 edges (interactions), some of which are known to be present in healing pathway. Notable proteins such as albumin, actin, collagen, fibronectin, histone, protein s100, vimentin, tubulin are abundant in its composition [11, 12]. Epithelial cell in amniotic membrane also secretes several growth factors and cytokines such as epidermal growth factor, vascular endothelial growth factor, keratinocyte growth factor, basic fibroblast growth factor, transforming growth factors alpha and beta (TGF-a and TGF-b), interleukin-8 (IL-8), angiogenin, dipeptidyl peptidase IV (DPPIV/CD26), serine protease inhibitor (serpin) E1, also known as type 1 plasminogen activator inhibitor (PAI-1), and insulin-like growth factors [3, 13–16].

2. Bovine amniotic membrane role in regenerative medicine and wound healing

Regenerative medicine is a branch of medicine concerned with developing therapies that regenerate or replace injured, diseased, or defective cells, tissues, or organs to restore or establish function and structure [17]. Regenerative medicine has become an integrated part in medicine and surgery. It has brought potential therapy possibilities with the aim to restore and improve the function of the damaged tissue or organ [18, 19]. There are many approaches to regenerative medicine, including stem cell, tissue engineering, organ transplantation and biomaterials. Biomaterials are either synthetic or natural material that are used for medical purpose or in contact with biological system. Biomaterials in regenerative medicine are intended to facilitate repair mechanism of wound. Usage of biomaterials in wound healing is applied on biological dressing. Biological dressing prevents evaporative water loss, heat loss, protein and electrolyte loss, and contamination and also permit debridement and develop granulation and epithelialization of wound bed [20].

Due to its structure as fetal ectoderm, amnion is considered as a physiological biological dressing [2, 3]. Amnion is favored due to optimal barrier against bacterial colonization and prevention of water loss [21]. Several studies on burn patients showed rapid epithelization of wound bed with minimal graft loss [21–23].

Ideally, the graft should be harvested from the same species (allograft). However, human amniotic membrane harvesting has several limitations. Fresh amnion is proposed to carry several infectious diseases such as hepatitis, AIDS, syphilis and tuberculosis [22]. There are also several difference for each amnion harvested from the same donor. The thickness is different at various site of membrane. The thickness could vary from 0.02 to 0.5 mm. This pattern also relates to its transparency and translucency of the membrane. Multiple attempts from the harvest also creates different thickness from donor harvested near the placenta and another distant from it [24, 25]. Differences acquired from different donors are suggested to be contributed from racial variation, duration of gestation, parity, gravidity, labor term and trial of labor before caesarian section. These factors influence prostaglandin and pro-inflammatory cytokines when transplanted to the recipient [24]. Difficulties in finding donor, long time storage issue also limits the supply [26]. Freeze dried human AM is proposed to be the solution for storage issue. Thomson and Parks in between 1979 and 1980 had studied the preparation of human amnion using sodium hypochlorite 0.025% solution and storaged it at −80° Celcius. There was no negative impact on its clinical benefits [23]. However, legal and religious issues still limit the supply of human amniotic membrane. Bovine amniotic membrane was proposed to help these shortcomings, providing stable supply.

Wound healing is characterized by sequential phases of inflammation, proliferation and remodeling [26, 27]. This process is mediated by many cytokines, growth hormones, and other mediators. Several numbers of studies were conducted resulting that bovine amniotic membrane may exert its therapeutic effects from inflammation to scarring process. Amniotic membrane contains proteinase inhibitors which inhibits migration of polymorphonuclear leucocyte cell to wound bed [28]. Polymoprhonuclear cell is one type of neutrophils that is extremely active on wound healing. Activated neutrophils produce several protease which help killing and degrading microbes. Neutropil-activated protease also breaks down extra-cellular matrix that in turn can debride the wound and facilitate cell migration. Nevertheless, excessive amount of neutrophils has negative impact on wound healing, by causing further tissue damage and inflammation [29]. A study by Kim et al. showed less polymoprhonuclear cell infiltration on amnion-membrane covered group thus promoting rapid healing and inhibiting proteolytic damage [30]. Another study by by Shimmura et al. proved that amniotic membrane attracts and traps inflammatory cells such as monocyte/macrophage, CD4(+) T cell and CD8(+) T cell which are responsible for cell apoptosis [31].

Bovine amniotic membrane also contributes on proliferation phase of wound healing. Abundant growth factors found on its membrane are responsible for the stimulation of proliferation [32]. Epidermal growth factor for instance works during proliferation phase (from day 4 postoperative) and promotes wound healing by increasing the rate of epidermal proliferation and also accelerating wound contraction level related to myofibroblast proliferation and collagen deposition [33]. Insulin growth factor, combined with Platelet derived growth factor and fibroblast growth factor increases the proliferation of fibroblast [34]. Fibroblast is crucial on wound proliferation by creating extracellular matrix and collagen structure for wound bed, as well as contracting the wound. Once fibroblast has migrated into the matrix, it changes its morphology and synthesize granulation tissue components [35]. amniotic membrane also has anti-bacterial peptides which makes it ideal for cell proliferation. Expression of inflammatory molecule suggested that amniotic

membrane is one part of the barrier to progression of infection [36, 37]. Bovine amniotic membrane collagen formation is also similar to human amnion according to its viability, diffusion formation and degradation [38].

Proliferation phase is also characterized by angiogenesis, granulation and epithelization. Each of these steps could also be influenced by amnion application. Matrix metalloproteinase (MMP) is one of growth factor expressed on amniotic membrane. It has been implicated in invasive cellular growth [3, 39]. A study by Jeong et al. showed that increased expression of membrane-type matrix metalloproteinase enhanced the activation of MMP-2 and invasion and migration of endhotelial cells which affect the induction of capillary tube formation [40]. Amnion cells secreted substantial amount of angiogenic factors including HGF, IGF-1, VEGF, EGF, HB-EGF and bFGF [41]. However, there are some studies about in vitro anti-angiogenic effects of amniotic membrane. Faraj et al. found that AM conditioned medium reduced proliferation and angiogenesis. This result was proposed to be induced by thrombospondin and tissue inhibitors of metalloproteinase (TIMP 1) and 2 [42–44].

Amniotic membrane also promotes granulation and epithelization. Analysis by Piscatelli et al. from in vitro model showed that wound contraction in fetus was influenced by combination of pro-contraction transforming growth factor-ß1 and anti-contraction epidermal growth factor [45]. Rapid epithelization was histologicallhy confirmed on bovine amniotic membrane-treated wound with thicker collagen bundles. Keratinocyte migration was also observed on wound bed whereas immunohistochemistry staining for angiogenesis and fibroblast were consistent for proliferation phase [26]. The connective tissue of amniotic membrane contains laminin, fibronectin and collagen, which are the main components of basal membrane. A case by Martinez et al. showed that spontaneous epithelization on epidermolysis bullosa was completed in seven days [46].

Other notable clinical effects of bovine amniotic membrane observed are anti scarring, fluid permeability control and tensile strength property. Anti scarring is promoted by anti-inflammation effect of AM and hyaluronic acid found on its membrane [47, 48]. Fluid control on wound healing is essential. Extravasation of fluid in wound contributes in creating wound exudate. The exudate is a marker of the chronic state of injury. Exudate also creates an environment favorable for bacterial proliferation [49]. Amniotic membrane structure remains intact after sterilization. Clinical study by Rejzek et al. has reported heat, fluid and electrolyte loss prevention by amniotic membrane. Oxygen permeability in amniotic membrane was demonstrated by Yoshita et al., all contributing to accelerated healing process [50, 51].

3. Application of bovine amniotic membrane in surgery

Coradetti et al. demonstrated that mesenchymal stem cells could be derive from bovine amnion. Both amnion and amniotic fluid are capable of differentiating into ectodermal and mesodermal lineages. This study further showed the capability of osteogenic, chondrogenic, adipogenic, and neurogenic stem cell usage for bovine amniotic membrane [52]. Thus, bovine amniotic membrane could be used for many surgical applications.

4. Ophthalmic surgery

The first use of amniotic membrane for ophthalmology was documented by de Rotth back in 1940. The conjunctiva defect caused by symblepharon was treated with fetal amniotic membrane. The graft was fixed to the tendon of rectus muscle

and was taken in all cases. The study showed that AM has transformation property toward conjunctiva tissue [53]. Since then, there have been many applications for ophthalmic disease ranging from ocular burn, corneal defects, retinal problems, strabismus, and neoplasia. However, most of them used human amnion [54–60].

Proteins expressed on bovine amniotic membrane showed to be abundant in human cornea: including keratocan, decorin, lumican, TGF-β-induced protein ig-h3, and albumin. These proteins are responsible for corneal healing pathways. Numerous signaling pathway responsible for corneal healing are also revealed in the membrane. Selected pathways include integrin signaling pathway, Cytoskeletal regulation by Rho GTPase, Ubiquitin proteasome pathway, WNT signaling pathway, epidermal growth factor (EGF) receptor signaling pathway [11]. Corneal healing was demonstrated on canine corneal erosion with significantly higher proliferation [61].

5. Plastic and reconstructive surgery

Bovine amnion function as biological dressing holds great potential in reconstructive surgery. There has been several clinical studies including bovine amnion membrane for burn patients [62–64]. Rao published a study in 1981 regarding the use of bovine amnion on burn and pressure ulcer patients. The study revealed biostatic ability to control infection and faster granulation process [6]. Another study by Zhu et al. showed significant difference of burn wound treated with bovine amnion compared with vaseline gauze dressing as control. The different results studied were healing time, infection ratio and residual burn wound [62]. Bovine amnion as burn biological dressing was also proven having similar efficacy as human amnion. A study by Park et al. displayed similar histological grading, epithelization rate and infection rate [22].

Ablative laser used for removing superficial skin has notable adverse effect even though its efficacy is better than non-ablative laser. Adverse effect such as postoperative erythema is caused by complete elimination of epidermis and upper dermal layer [65]. Bovine amnion membrane was found clinically effective in reducing erythema due to its anti-inflammatory mechanism [26].

6. Head and neck surgery

Amnion is also applied in head and neck surgery. Although, some of them used human amnion. One of such use was documented as the use of amniotic membrane compared with collagen membrane, in a study conducted by Munoyath consisted of twenty patients with facial soft tissue injury with whom all patients had either single or multiple soft tissue loss all over the face. The size of the wounds ranged from 7 mm × 10 mm to 80 mm × 150 mm and depth of the wound ranged from 2 to 5 mm. the results showed pain score of greater than 3 was observed in 50% of the patients in Amnion group and in 80% of the patients in Collagen group. The average time for appearance of healthy granulation tissue over the wounds that were treated with Amnion dressing was 5 to 9 days and for Collagen group was 7 to 12 days. Though vascularity was not compromised in both the groups, the height of the wound at 3-month follow up showed a clinically significant difference (100% patients in AM group had flat wound whereas only 80% showed normal wound height); though statistically not significant [66].

In recent years, research of amniotic membrane in head and neck surgery, especially bovine amniotic membrane, has increased. The use mostly relates to wound dressing. Further research could evaluate the use of bovine amniotic membrane

in head and neck surgery in the years to come. The amniotic membrane compared with collagen membrane, with both materials combined with deproteinized bovine bone mineral was compared in clinical trials by Kim for the treatment of periodontal inflammations. Both the use of amniotic membrane and collagen membrane combined with deproteinized bovine bone mineral improves the condition of periodontium. The amnion did not cause a significant gum recession. Another use of amniotic membrane for oral cavity problem was studied for temporomandibular joint ankylosis, which is a serious condition, mainly due to injuries responsible for the reduction of mandible functionality [67, 68].

A bovine amniotic membrane study for facial abrasions was done in a Korea, comparing the use Amnisite BAtm with foam and gel dressings. The study demonstrated all patients were well healed completely after appliance of dried bovine amniotic membrane or foam dressing without any complication. However healing time for patients treated with dried bovine amniotic membrane was significantly shorter and no significant difference between the two groups regarding treatment costs, scar formation, skin elasticity or moisture content was noted. This study demonstrate the potential practical clinical use of bovine amniotic membrane as a facial soft tissue trauma as one of potential dressing [69].

7. Neurosurgery

In the field of neurosurgery, autologously harvested amniotic membrane has been used to repair duramater defects in myelomeningocele. Although the human cranial neurosurgery applications of amniotic membrane have not been thoroughly investigated, an in vivo rat cranial surgery model demonstrated that human xenograft amniotic membrane was efficacious and had an adequate safety profile. Eichberg put forward their retrospective pilot study about the use of allograft amniotic membrane for the augmentation of dural repair in craniotomies. The reported rates of postoperative CSF leaks differ among studies and craniotomy locations; leaks may prevailed as many as 4–17% craniotomy for posterior fossa lesions. In 122 craniotomies, including 18 craniotomies for posterior fossa lesions, none were complicated by postoperative CSF leaks. These results suggest that amniotic does not contribute to the increased risk of CSF leaks. Further, the interpretation of the data is complicated by the fact that the patients in the study received a sheet of bovine collagen dural substitute layered on top of the dehydrated amnion membrane; thus, the outcomes may be due to both materials. While this retrospective pilot study does not prove the superiority of dehydrated amnion over other dural adjuncts, or the efficacy of use, they demonstrate that it has an adequate safety profile with no complications directly related to its use in closures for craniotomies. They also report very low CSF leak rates and infection rates, particularly in craniotomies for infratentorial lesions [70–72].

8. Pediatric urology: the prospect of urethral reconstruction using bovine amniotic membrane

In the field of urology or pediatric surgery, uroepithelial reconstruction for several pathology is challenging. There is no synthetic material that is considered ideal as a substitute for the urethra and there is no research that firmly determine a good synthetic material to replace urethral defects. Strictures still found after the transplantation of acellular scaffold was also reported [73]. Therefore, the use of cell-seeded scaffold is proposed to be a better material used in urethral reconstruction. Bovine amniotic membrane has unique properties including anti-adhesive effects, bacteriostatic

properties, wound protection, pain reduction, and epithelialization effects. Another characteristic of amniotic membrane is the lack of immunogenicity [26].

Amniotic membrane also has some advantage compared to other allographs, such as bladder mucosa, buccal mucosa, and also appendix tissue. First of all, amnion harvesting does not need extra surgery unlike other organ. Postoperative care is simpler with shorter hospital time. However, there is technical difficulty in handling the membrane. Due to the thinness of the amniotic membrane and the release of the amniotic layer to the chorion, there is a higher risk of perforation or tear or separation of the two layers during surgery [74].

Bovine amniotic membrane has been studied mostly on animal studies with promising effects. One experimental study for urinary bladder reconstruction by Bakhtiari in 2000 was using fresh and formalin based bovine amniotic membrane for canine. Urinary bladder and urethra share similar histology. Both are consisting of epithelium on the lumen surrounded by rich collagen connective tissue and muscle layer. Both are responsible for maintaining structural integrity of the organ and transporting or expelling the urine [75]. The surgical procedure was conducted by 5 cm resection from the cranial bladder. Postoperative graft using two types of bovine amniotic membrane was observed. Graft site observations on postoperative days 30 and 60 showed adhesion at the graft site (100%), the graft floating within the bladder lumen (40%), good graft adhesion to the bladder, and no evidence of leakage or fistulation. Histopathological examination revealed regeneration of uroepithelial tissue and smooth muscle at the graft site. In addition, congestion, edema, and inflammatory cell infiltration were also seen in two cases. According to this study, despite complications such as infection, release of amnion from the bladder, "less than normal" distension and adhesions of the bladder at the graft site, it can be argued that fresh and preserved bovine amnion acts as a scaffold for the repair of canine bladder defects. The regeneration of the urothelium and the presence of microscopic smooth muscle and the important complications that may occur from enterocystoplasty can encourage the use of bovine amniotic membrane for bladder reconstruction. However, long-term studies are still needed to assess other clinical and laboratory findings before measuring for clinical use [76].

In another study, Shakeri demonstrated the ability of the human amniotic membrane to induce epithelialization in experimental study on rabbits by reconstructing the urethra using the human amniotic membrane. The evaluation was conducted after 30 days post-operative. The result showed re-epithelialization of urethral without inflammation and tissue loss. The author also concluded that amniotic membrane is an inexpensive, easy, and biodegradable graft with very little antigen effect which seems to be the ideal solution for urethroplasty [74].

Amniotic membrane could be used as potential source for stem cell. Ghionzoli and Chung showed that it could be differentiated into smooth muscle and urothelial cell, both which are becoming integral parts creating urethral tissue [77, 78]. Despite using human amniotic membrane, some preclinical studies have also explored urological applications. Iijima demonstrated that amniotic membrane could successfully be used for bladder augmentation in rats. Human amniotic membrane-augmented bladder revealed regeneration of urothelium, detrusor smooth muscle, and nerve fibers within 3 months post-operatively. Bladder capacity was also found to be normal within 4 weeks post-operatively [79].

Pusateri evaluated placental membrane grafts for urethral replacement in rabbit model. The procedure consisted of mobilitation of urethra, dorsal urethrotomy and graft placement. Dorsal onlay urethroplasty was performed afterwards. Observation after 3 month showed urethral patency in all rabbits. On pathologic examination, urothelial cell replacement was observed in all rabbit without malignant transformation. Urothelium was intact and circumferentially normal in all sections of graft bed. On cystourethroscopy, there was no strictures, fistulas or massess reported [80].

Wang et al. looked further to this idea, namely using the collagen scaffolding of amniotic membrane as potential regenerative material in urethroplasty. The authors separated basement layer of amnion to retrieve denuded human amniotic scaffold. Rabbit urethral epithelial cell was inoculated on its surface and the response showed mild immune reaction with no rejection. This maximizes the biocompability of amniotic membrane making it potential biomaterial for urethral reconstruction [81]. Gunes took another approach by combining amniotic membrane and buccal mucosa for penile urethral reconstrucion in rabbit model. Both membrane were obtained from rabbit. Gunes compared whether buccal mucosa, amniotic membrane or both might be useful in urethroplasty. The best result of epithelial transformation was shown in combined group after 8 weeks with no complication regarding fistula or dehiscence observed [82].

Hariastawa et al. compared both bovine and human amniotic membrane for reconstruction of urethral defect in rabbit animal model. His study aimed to discover the difference in mucosal integrity between both groups. Epithelium layer was formed in both group with no significant ddifference of postoperative mucosal grading on day 7,14 and 28 postoperatively. Authors concluded that bovine amniotic membrane could be used as good, cheap alternative in urethral defect reconstruction [83]. In another study, Hariastawa used dried amniotic membrane scaffold with adipose derived-mesenchymal stem cell seeding for rabbit penile urethral reconstruction. Adipose cell was cultured from rabbit neck and mixed with fetal bovine serum. Viability of stem cell was tested before the surgical procedure. The urethral wall was cut transversely before the scaffold that had been seeded with stem cells was implanted as urethral graft. Urethroplasty was done afterwards. Post-operative clinical observation showed urethral integrity alongside the defect. Urethral specimen was harvested on day 28 post-operatively. The specimen was then observed using fluorescene microscope. Neovascularization and best epithelial transformation was seen in combined amniotic membrane and adipose-derived mesenchymal stem cell seeding group. The promising result showed that stem cell could be used as adjunct treatment for amniotic membrane application [84].

Although limited to human amniotic membrane, clinical researches have been accepted. The first clinical report regarding the use of human amniotic membrane for anterior urethral defect repair was reported by Razzaghi et al. This pilot study included patients with previous hypospadia repair [85]. Hypospadias repair remains a challenge due to many anatomical variation of the pathology, surgical techniques and comorbidities of patient. Complications vary from dehiscence, stenosis to fistula formation. Secondary or salvage procedure were often needed for failed primary repair [86, 87]. After reconstruction of neourethra and proper hemostatis from urehtroplasy, the allograft was used to cover the suture lines. Observation between 7 to 18 moths post-operatively showed no long term complications. Amniotic membrane graft was proposed as an applicable, low-cost, biodegradable cover for second hypospadias repair [85]. Oottamasathien et al. proposed that amniotic membrane could be used for reducing complication rate, particularly from high re-operation rate of hypospadias. The underlying premise is to provide a barrier layer with robust source of tissue, vascular growth factors and anti-inflammatory environment for soft tissue healing [88].

9. Summary

Potential future application of bovine amniotic membrane could be explored widely. Broad number of biological properties found in amniotic membrane described above presents future studies. Preclinical and clinical researches could be used for basic scaffold for other applications. Combination with other biomaterials would be considered further.

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