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Ruminants

The Husbandry, Economic and Health Aspects

Edited by Muhammad Abubakar

RUMINANTS - THE HUSBANDRY, ECONOMIC AND HEALTH ASPECTS

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http://dx.doi.org/10.5772/intechopen.68639
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Notice

First published in London, United Kingdom, 2018 by IntechOpen
eBook (PDF) Published by IntechOpen, 2019
IntechOpen is the global imprint of INTECHOPEN LIMITED, registered in England and Wales, registration number: 11086078, The Shard, 25th floor, 32 London Bridge Street
London, SE19SG – United Kingdom
Printed in Croatia

British Library Cataloguing-in-Publication Data
A catalogue record for this book is available from the British Library

Additional hard and PDF copies can be obtained from orders@intechopen.com

Ruminants - The Husbandry, Economic and Health Aspects
Edited by Muhammad Abubakar

p. cm.

Print ISBN 978-1-78923-048-2

Online ISBN 978-1-78923-049-9

eBook (PDF) ISBN 978-1-83881-336-9

Meet the editor

Dr. Muhammad Abubakar, a scientist from the National Veterinary Laboratory, Islamabad, Pakistan, has more than 15 years of experience in various fields of veterinary sciences. His main expertise is on the transboundary animal diseases (TADs) both on national an international level. He has worked on national and international projects for the establishment of diagnostic laboratories for TADs in the country. He has also conducted various trainings for field as well as laboratory staff. He has published numerous research papers, review articles, and book chapters on different areas of veterinary sciences especially on TADs (Avian influenza, FMD, PPR). Dr. Abubakar is the coeditor of the book *The Role of Biotechnology in Improvement of Livestock* by Springer Publisher, Germany. He is currently working as an editor in chief of two journals in the area of veterinary sciences (*Research journal for Veterinary Practitioners* and *Veterinary Sciences*: *Research and Reviews*).

Contents

Preface

It is very essential to understand the recent advances in the ruminant science to recognize and control diseases and disorders in these animals. These findings are also critical for enhancing production and reproduction in ruminants. Openly available, *Ruminants - The Husbandry, Economic and Health Aspects* provides a concise introductory chapter and details about the main three aspects of ruminants' science and production. This is the first edition of the book, so it covers the introductory level of topics, which are written specifically for veterinary and animal health students and scientists.

In the introduction section, there is a detailed description about the importance of ruminants for the world economy and food security. Way forward and future concepts are also covered under this section.

In the "Husbandry and Economics" section, the focus is given to the use of silage for climate resilient small ruminant production and similarly beef cattle production in semiarid regions. Another important area covered in this section is the twin calving production in dairy cows, which is a revolutionizing area in this science. These topics provide an excellent introduction to husbandry and production techniques and their application to ruminants' production and economics. The book is self-contained, with everything needed to understand the importance of ruminants' husbandry and economics.

In the "Animal Health" section, the book provides an emerging area of concern related to ruminant's health, i.e., mycotoxins that affect severely their health, and a detailed chapter is given which covers all major aspects including control measures. An introductory and up-to-date overview on the use of serum proteins for the laboratory diagnosis of animal health issues is given. It provides an excellent introduction to anyone interested in acquiring a basic understanding of lab diagnosis based on serology.

Throughout the book, examples are drawn from the field of veterinary and dairy science, and a clear account is provided on the importance of the different areas of ruminant's health. The various concepts are clearly and concisely expressed. The writing is generally clear, realistic, and quite positive on various aspects for easy understanding.

The book coverage includes the following main areas:

- Introduction
- Husbandry and Economics
- Animal Health

Each book section comprises chapters from renowned experts from the area and gives readers a unique opportunity to explore the topic.

I would like to express my gratitude to all the contributors of this book including the authors of the accepted chapters. My special thanks go to the Publishing Process Manager, Ms. Romina Rovan, and other staff of IntechOpen publisher 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 in the fulfilment of this project.

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Introduction

Chapter 1

Introductory Chapter: Ruminants—The Husbandry, Economic, and Health Aspects

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Abdul Kabir and Shumaila Manzoor

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.76064

1. Introduction

Ruminants have a valuable role in sustainable agricultural systems and provision of food to human beings. They play a pivotal role in converting vast renewable resources from rangeland, pasture, and crop residues and/or other by-products into food edible for humans. Grazing ruminant animals is an efficient way to produce food for humans. The need to maintain ruminants to utilize these humanly inedible foodstuffs and alter them into high-quality foods for human consumption has been a characteristic for several thousand years. In fact, dairy cattle and goats are quite exceptional in being extremely efficient in converting plant-based protein/energy sources into high-quality animal fats and proteins. It is convincing that ruminants are essential components in food production systems now and in the future.

2. Livestock value

Livestock is an important asset throughout the world, with a value of at least $1.4 trillion. This sector is subdivided in long market chains that provide employment for almost 1.3 billion people worldwide and livelihood of 600 million poor smallholder farmers in the developing world directly depends on livestock [1]. Ruminants fulfill numerous roles, ranging from providing manure, milk, meat, and draught power. Animal protein is one of the major parts of the daily food supply. Globally livestock products contribute 33% of daily protein consumption in the shape of eggs, meat, and milk. The demand of livestock products is increasing day by day due to rapid urbanization and population growth [2].

In developed countries, animal diseases adversely affect the economy of livestock farms, businesses, and animal production sectors, whereas in developing countries, there are additional menaces of food scarcity and capital reduction [3].

In developed countries, during the last few decades, there has been a decrease in livestock diseases due to the increase in vaccine's quality and quantity, more effective drug development, and advancement in diagnostic technologies. At the same time, the emergence of new diseases such as avian influenza H_5N_1 became a threat for whole world [4]. In developing countries, there have been comparatively less changes in the incidence rate and prevalence of diseases of livestock. Maladministration and husbandry practices can increase the susceptibility to parasites and pathogens. If any young animals die of disease at an early age, this decreases profit [5].

3. Ruminants health and economics

Outbreak of any disease adversely affects the livestock production starting a process that progressively leads to low production and little profit. Animal diseases in livestock are mostly multifaceted and affect all the persons involved in food chain starting from livestock owner to the livestock product consumer. These diseases affect the economy through various ways, including decreased production, increase cost of treatment, market disturbances, a ban on the transportation of livestock products, limited tourism, and prevention and control expense [6].

If we take an example of foot and mouth disease (FMD), a disease of low mortality, but the worldwide impact of FMD is massive due to a larger number of animals affected by disease [7]. The losses due to FMD can be subdivided into direct losses because of decreased production and indirect losses due to expenses of FMD control and limited access to markets [8]. It is estimated that outbreaks of FMD in FMD free countries and zones can result in loss of >US$1.5 billion per year. FMD endemic countries suffer annually between US$6.5 and 21 billion due to production losses and vaccination [9]. Foot and mouth disease outbreak in the UK in 2001 resulted in the slaughter of 6 million animals [10].

Mastitis is another major problem of dairy animals that negatively affects the production. This directly lessens the net profit due to treatment expenses, reduced milk production, and quality of milk. Indirect impacts of disease include decreased fertility, increased culling rate, and rarely mortality [11]. Globally, published data regarding the economic losses of clinical mastitis depict the loss from €61 to €97 per cow on a farm, depending upon preventive practices. For example, in The Netherlands, economic losses due to clinical and subclinical mastitis varies from €17 to €198 per cow per year [3].

Bovine respiratory disease (BRD) is the most common disease among the feedlot cattle. BRD causes an estimated $800 million to $900 million annually in economic losses from death, reduced feed efficiency, and treatment costs [12]. Peste des petits Ruminants virus causes large economic losses each year due to high mortality and morbidity rates in the infected sheep and goats and outbreaks were more severe in goats than sheep. Global estimated impact due to this disease is in between US$1.4 and 2.1 billion [13].

4. Control and eradication of diseases

For prevention and control of infectious diseases, strict biosecurity measures, quarantine, isolation of infected and disease suspected animals, effective disease surveillance, monitoring and networking programs with suitable vaccination, and treatment strategies play a key role [14]. Routine farm practices should include measures to decrease spread of transmissible diseases, for example, by improving hygiene, keeping newly purchased animals in the quarantine, and establishing coordinated, sustained surveillance for diseases that can spread beyond borders of countries and continents [15]. Control of any disease is varying value that depends upon the country and region and needs annual expenses [16]. All most all the disease can be controlled by following simple procedures such as quarantine and vaccines. The eventual feat of control of any disease is the eradication of disease. But it is not compulsory that a disease that can be controlled can also be eradicated.

Proper cleaning and disinfection is a critical step in limiting the fecal-oral transmission cycles of pathogens that are mostly present in the feeding or treatment equipment or fomites [17]. The first step is a systematic cleaning to remove organic material before applying disinfectant. Everyday vigorous scrubbing can avoid the development of biofilms, which act as a shield for microorganisms against disinfectants.

Adequate ventilation is imperative to the health of housing animals. Sufficient ventilation in a walled housing not only removes infectious aerosol pathogens but also reduces humidity [14]. Decreased humidity decreases the survival time of airborne and surface-borne infectious agents. In case of disease outbreak bedding materials from infected animals, feed stuff, excretory and secretory products including dung and urine, and clothing of people working in infected animal houses should be destroyed properly [17]. Biosecurity refers to the management practices that decrease the contact of infectious pathogens in animals. Adoption of biosecurity measures can prevent the incidence of infectious animal diseases. Closed door policy is a pivot point of biosecurity at any farm [18].

Vaccination has been a practical approach for the control and eradication of several infectious diseases worldwide. Effective control measures along with proper vaccination can lessen the incidence rate of the different animal diseases [19]. Vaccination is used to progressively decrease the burden of infection until either eradication becomes certain or culling policy becomes an economically feasible option [18].

Many factors interrelate to lessen the immunizing efficacy of vaccination programs such as low quality vaccines, improper vaccine storage, and immune status of animals [20]. Recent molecular techniques such as development of subunit DNA vaccines, recombinant vaccines, and non-pathogenic virus-vectored vaccines lead to the production of more efficient and safer methods of immunization [16].

OIE pathway to control any disease includes preliminary surveillance for that disease, followed by mass vaccination to control the disease, and then again serological surveillance to monitor disease prevalence. These approaches push the country on the way to free from diseases that result in the declaration of provisional absence of disease [15].

Constraints in controlling the diseases should be resolved to encourage the eradication of diseases which would ultimately reduce the economic loss. Basic limitation for the eradication of any livestock disease is the cost of eradication. In developing countries of Asia and Africa, where the diseases are endemic control by vaccination of animals is a feasible option than culling of seropositive animals [21]. Due to limited funds, mass vaccination for controlling any disease is also a serious issue. Another limitation is that veterinarians are unable to collect the samples due to the lack of technical expertise, deficiency of facilities for sample collection, and preservation and transportation to the adjacent laboratory for accurate diagnosis [14].

5. One Health concept

The One World, One Health theory represents that the health of all living things present on this planet depends upon the health of other living things [22]. To survive in this world now, we should consider the planet as a dynamic system, in which the health of each component is linked and reliant on others [23]. If we want to control human diseases, we should also consider the diseases of animals. Nearly 60% of human infectious diseases is from animal origin and 75% of the emerging infectious diseases of humans reported during the last 30 years is of animal origin too [24].

Several emerging infectious zoonotic diseases have arisen as a threat to food supply and the control of these diseases needs the collective expertise [25]. As human population is growing day by day, interaction between the people and wild animals is also increasing. This exposes human to diseases [26]. Ebola and AIDS are two major examples that possibly transmitted from chimpanzees to humans [24]. One Health gives a noteworthy chance to veterinarians to cooperate with human medical experts, wildlife, and environmental health professionals for the greater good [27].

6. Conclusion and future perspective

The animal diseases should be given serious consideration and advanced research facilities should be established. For successful control of livestock diseases, epidemiological forecasting, accurate and early diagnosis, safer and quality vaccines availability along with adequate infrastructure facilities for cold storage and transport facilities are required. This will increase the livestock production, which eventually results in alleviation of poverty in the rural areas. We must adopt such husbandry practices so that we can keep livestock in such a way that it is best for individuals, communities, and the planet.

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Section 2

Husbandry and Economics

Chapter 2

Silage for Climate Resilient Small Ruminant Production

Artabandhu Sahoo

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.74667

Abstract

Climate change impact on livestock, especially due to impact on agriculture and ensuing shortage of feed resources and its quality, will have a profound effect on growth, milk production, reproduction, metabolic activity and disease occurrence. Small ruminant feeding and nutrition research should therefore be tailored in line with climate resilient agriculture and farming systems. Seasonal feed scarcity is a concurrent problem that farmers usually face besides natural calamities like drought, flood, cyclone, earthquake, etc., and it has a significant impact on small ruminant productivity. Silage making is an effective and common method of forage preservation and also a form of treatment to occasionally retrieve the underutilized pastures for better acceptability, degradability and utilization. Demand for conventional crop (principally maize) outpaces its production, which stresses upon to find suitable, or even better, alternatives for silage making. This chapter deals with silage making from legumes, mixed forages, alternate forages and by-products from fruits and vegetable sector, TMR silage, phytochemicals role in silage making and livestock production, use of inoculants/additives in silages, the concept of therapeutic silage, novel microbial approaches to solving the problem of silage aerobic deterioration during the feed-out phase, animal and human health concern of deteriorated silages and production of designer animal produce from innovative silages.

Keywords: silage, small ruminant, feed scarcity, nutrition, productivity

1. Introduction

Seasonal shortages in feed supply are major constraints to increasing ruminant productivity in developing countries. Small ruminant feeding and nutrition research should therefore be tailored in line with climate resilient agriculture and farming systems. The calamity of

climate change should be converted into an opportunity for developing and spreading climate resilient small ruminant farming and production systems. Natural pastures, crop residues and indigenous fodder trees are the main feed resources for ruminant livestock. But, due to seasonal fluctuations in the availability and quality of these feed resources, intake of energy, protein and some essential minerals by most ruminant species fall below their maintenance requirements resulting in 'under-nutrition' and low productivity in most animal production systems [1]. The leftover natural pastures, particularly the abundantly grown monsoon grasses/herbages that get matured (lignified) and dried have limited intake and characterized by low nutritive value, digestibility and utilization. In dealing with the rainy season crop harvest, and due to the difficulties in hay storage, ensiling is considered as one of the preferable preservation techniques especially with the greatest potential for protein-rich foliage.

Silage making is an effective and common method of forage preservation and also a form of treatment to occasionally retrieve the underutilized pastures for better acceptability, degradability and utilization. It is universally agreed that silage making is one of the principal approaches if feed and nutrition is to be ensured round the year. In the rainy season, there is an abundance of grass, while it becomes scarce in the dry season, and therefore, silage production in the tropics has been established as a sustainable means of supplementing feed for ruminants in the dry scarcity periods. Maize is observed to be the major crop for silage making, but as the demand for maize/corn outpaces its production due to current changes in global energy system for the production of biodiesel, coupled with increasing competition between animals and humans for this major food/feed item; it has become imperative to research into suitable, or even better, alternatives to this conventional crop for silage making. Low cost unconventional plant biomass offering promising nutrients may serve as an alternative, because of changing climatic conditions and lack of opportunity to cultivate fodder due to shortage of water resources. Hence, the perennial forage surplus obtained when the weather is favorable is recommended for storage as silage in order to meet the animal requirements throughout the year [2]. Alternate forage resources (browses and tree forages, field and crop wastes), succulent plant biomass (roots and tubers, cactus, fruits and vegetables co-products/wastes) and conceptualization of legume silage, mixed silage, total mixed ration (TMR) silage and their application would certainly expand the forage resource base and feed banking for ensuring nutritional input.

2. Climate resilient small ruminant production

Climate change leading to adverse changes in temperature, precipitation and sea level will disturb the food, water and livelihood security systems. The impacts of climate change on livestock are on its growth, milk production, reproduction, metabolic activity and disease occurrences. The indirect impacts include (i) scarce availability of water, pasture and other feed resources, (ii) health anomalies associated with modified/unknown vector-borne and parasitic diseases, (iii) competing environmental interaction with other livestock species. It is important to understand the small ruminant vis-à-vis other livestock responses to the changed

climatic environment and to analyze them in order to design modifications of nutritional and environmental management, thereby improving animal comfort and performance. In many countries, there is a scarcity of forage for ruminants feeding because of climatic conditions and shortage of water resources. The success of small ruminant rearing mostly depends on congenial macro- and micro-environments and the effectiveness of the ameliorating measures taken to reduce the stress factors. Adapting to climate change and reducing greenhouse gas (GHG) emissions may require significant changes in production technology and farming systems that could affect productivity. Globally, livestock contribute to 18% of the human-generated greenhouse gases, and the main components include methane (CH_4) produced by the belching of animals (25%), carbon dioxide (CO_2) by uses of land due to decomposition of organic substances (32%) and nitrous oxide (N_2O) due to spreading of manure and slurry over land (31%) [3]. One of the best ways of mitigating enteric methane emission could be improvement of the feed and forage of the ruminant animals to enhance the feed-conversion efficiencies in the production of a unit of milk or meat.

3. Silage making

Ensilage of forage crops has been practiced in one form or another for more than 3000 years. Several technical advances in silage making, such as multiple application of forage harvester, rapid and efficient silo packing, effective exclusion of air from silos, control of undesirable bacteria and use of silage additives (e.g. formaldehyde, glutaraldehyde, sodium hydroxide, sodium acrylate, urea, formic acid, etc.), and their application have expanded production and application of silage in livestock feeding. Emphasis has also been given on its qualitative enhancement due to increase in its usage in dairy and other ruminants. Ensiling forage enables preservation of succulent nature besides converting it to a form considered more utilizable by the ruminant livestock. Good silage is light brown in color, has a sour taste and pleasant acidic smell due to its lactic acid content, which make the product stable and can be kept for 6 months to 1 year, if required. This technology can be practiced round the year as and when any surplus plant biomass is available and yield better quality conserved forage to feed during scarcity.

Ideally, crops for silage are harvested at right stage, i.e. at 50% flowering, and then chopped into 2–5 cm pieces and left for wilting if needed to have moisture content not more than 60%. It is important to note that the influence of forage characteristics (epiphytic lactic acid bacteria (LAB), buffer capacity and sugar: buffer capacity ratio) on treatment effectiveness varied with DM content. Any additives (molasses, probiotic culture, etc.) can be added and mixed uniformly by spreading the chopped materials on a pucca (concrete) floor or polythene (plastic) sheet and then transferred to polythene lined silo pit (**Figure 1**) or plastic bags (**Figure 2**)/ drums (**Figure 3**) and compressed tightly in order to make it air free. It is kept anaerobically away from direct sunlight under the shade for 55–60 days under anaerobic condition. To make good quality silage, one must have quality assessment of the plant, microbial and environmental factors that influence the fermentation process and, ultimately, the nutrient value of the silage. It is essential to harvest forage at the right time, from the point of view of nutritional

Figure 1. Silage pits lined with plastic sheet to prevent seepage.

Figure 2. Packing of fodder in plastic bag for silage making.

quality, quantity available and climatic conditions, and then to store it properly to reduce losses. Silage made from grasses and cereals is dark yellowish green in color, while it is blackish green when made of legumes. A good silage is friable, non-sticky and free from mold/fungal growth and should have an acceptable and pleasant aroma (fruity odor) and mild acidic taste. It should have a pH < 4.5; the lactic acid should be higher than other acids with a low butyric acid content (0.2–0.5%) and ammoniacal N not higher than 9–15% of total N.

Farmers' friendly ensiling process has been developed in many countries for its wider adaptation, which is relatively simple, can be performed manually, is flexible in handling and feed-out according to needs and does not require much input. Dry tree forages, less palatable fallen tree leaves, less preferable stovers and mature crop residues can be mixed with high moisture

http://dx.doi.org/10.5772/intechopen.74667

Figure 3. Silage making in plastic drums.

containing cactus, azola, residual/leftover vegetables and fruits to prepare mixed silage of desired quality with proper balancing for appropriate moisture, degradable and water soluble carbohydrates (WSC) and N-fractions. The preservation of forage crops as silage depends principally on the production of sufficient acid to inhibit activity of undesirable microorganisms under anaerobic conditions [4].

3.1. Suitable crops for making silage

Forage crops should be harvested for silage making from flowering to milk stage of the crop. Forage characteristics, viz. type of forage, maturity, DM and WSC content, at the time of ensiling influence the ease of ensiling and ultimately the quality of silage. Cereals, in general, are easier to ensile than legumes or grasses, because of their lower buffering capacity and high WSC content. As forage matures from the vegetative stage into reproductive stage (i.e. heading for cereals; flowering for legumes), stems and leaves become more lignified, and the digestibility of these plant components declines. Several factors influence the rate of maturation of a crop including variety, moisture level, temperature, nutrient stress and time of season. Thus, optimal timing of harvest usually encompasses a compromise between DM and nutrient yield. DM content of forage tends to increase with advancing maturity, but silage DM can also be increased by wilting a less mature forage in the field prior to ensiling. Grass family crops are more suitable for making silage because of higher sugar and WSC, e.g. jowar (*Sorghum bicolor*), bajra (*Pennisetum glaucum*), maize (*Zea mays*), guinea grass (*Panicum maximum*), cenchrus grass (*Cenchrus ciliaris*, *C. setigerus*), sudan grass (*Sorghum sudanense*), oats (*Avena sativa*), barley (*Hordeum vulgare*), napier (*Pennisetum purpureum*), etc. [5]. Making silage only from leguminous crops like berseem (*Trifolium alexandrinum*), Lucerne (*Medicago sativa*), soybean (*Glycine max*), lobia/cowpea (*Vigna unguiculata*) is not advisable since they contain high moisture and less carbohydrate. Hence, they are mixed with grasses for making quality silage.

3.2. Ensiling legume crops/fodder

Current restrictions on the use of animal-based protein supplements coupled with increasing demand for soya protein concentrates put pressure on the livestock farmers and researchers to

consider alternative home-grown protein-rich forage crops as supplements to grass silage for sustaining production. Ensiling legumes is a good way of providing a cheaper, non-animal-based and traceable home-grown protein that may improve the efficiency of production system in any livestock farms. Also, legume silages with low DM and WSC contents are generally more resistant to aerobic deterioration than cereal silages [6]. There are also some unidentified microbial inhibitors that prevent the growth of spoilage microorganisms [7]. But, most legumes undergo butyric acid fermentation when ensiled without additives at low DM content due to low WSC and high buffering capacity [8]. Furthermore, severe degradation of proteins may devalue the protein quality due to inefficient N utilization.

Besides, stage of maturity and DM content of the crop at ensiling, wilting and rate of drying markedly reduce proteolysis in the silo [9]. Rapid and extensive wilting (DM > 500 g/kg) improved protein value and reduced CP degradability. Moreover, due to the reversible protein-binding properties of tannin, species containing tannin have shown to undergo less protein degradation during ensiling than that do not contain tannin. Since protein degradation in the silo is widely recognized to be the most limiting factor in legume silage, intrinsic protein protection by mixing tannin-containing forages may contribute to reducing the rate and the extent of NPN formation in silages, thereby improving N usage. Fraser et al. [10] compared the nutritive value of a range of ensiled forage legumes from late second-cut lotus (*Lotus corniculatus*), first-cut sainfoin (*Onobrychis viciifolia*) and both early and late second-cut red clover (*Trifolium pratense*) and lucerne and found high intake potential of all the legume silages. The taniferous lotus silage recorded higher intake and N utilization compared to less/non-tannin legumes, clover and lucerne, while low N digestibility appears to limit the nutritional value of sainfoin. In silages made from the beginning of ripening stage, where most of the protein was localized in the seed, the level of proteolysis was reduced and a good fermentation was observed in peas ensiled after a short wilting period [11]. Possible approaches include the adoption of harvesting techniques that reduce field wilting time, the use of protein protection agents during ensiling such as tannins or the choice of natural tannin-containing legume species.

3.3. Alternate plant biomass for silage making

Industrialization of food production has produced large quantities of food wastes, viz. (i) crop waste and residues, (ii) grain and legume by-products; (iii) distillery and brewery by-products; (iv) fruit and vegetables by-products; (v) sugar, starch and confectionary industry by-products and (vi) oil industry by-products. Fruit and vegetable processing by-products/co-products are promising sources of valuables such as phytochemicals (carotenoids, phenolics, flavonoids), antioxidants, antimicrobials, vitamins or dietary fats that have favorable technological activities or nutritional properties and have traditionally been used as feed ingredients, and their effect on animal performance has been extensively studied [12–14].

Key determinants while selecting unconventional resources: Moisture content is the most important factor in silage making, with a recommendation at 65–75% [15] depending on the means of storage, the degree of compression and the amount of water that will be lost in storage. Effluent is produced when moisture is above 75%, with the amount of effluent increasing with increasing silo height due to increasing pressure. In general, forage with high moisture

content makes sour silage. Additionally, the critical pH value for clostridial growth varies directly with the moisture content of the plant material, and unless WSC levels are exceptionally high, ensiling wet crops encourages clostridial fermentation, resulting in high losses and reduced nutritive value. Moreover, many high CP legume foliages can be difficult to ensile successfully, because they tend to have low WSC, high buffering capacity and low DM content when directly harvested [8].

The characteristics of good silage should have pH values of 4.2 or less, NH_3-N contents <100 g/kg DM and high lactic acid contents. Legumes that have low WSC and high buffer capacity do not produce good quality silage [8]. High N-containing leguminous fodder also includes tree forages and browses that are rich in plant secondary metabolites (PSM). Many times these phytochemicals may become adverse to fermentation process (e.g. antimicrobial effects of alkaloids, essential oils, etc.) and put forth additional challenges to step up microbial fermentative activity. Further, degradation of protein during ensiling process produces volatile organic acids with higher pKa values, and thus, the silages may have higher pH, unfavorable aroma, less aerobic stability and greater spoilage. Further, the relationship between weekly growth rate and change in quality parameters is differed among species and functional groups, i.e., grasses and legumes, and therefore, quantifying the impact of delaying the harvest date of grass-legume mixtures and assessing the relationships between productivity and components of feed quality are important.

3.3.1. Phytochemical-rich forages

Tannins in ruminants can induce beneficial effects attributable to tannin-protein complexes, which lead to increased rumen escape of dietary protein and increase in microbial protein outflow. High N fertilized grasses are more degradable, which are hydrolyzed extensively during ensiling and are rapidly degraded in rumen resulting in more excretory loss. Decreased proteolysis and slower fermentation of proteins and NPN are particularly important in silages for uniform N availability to ruminal microbial synthesis and thus optimizing its usage. Forage legumes with PSM are considered to be less susceptible to proteolysis than other legumes, which improve silage quality. The legume sainfoin has been shown to contain tannins of particularly beneficial composition for ruminant nutrition. Adding tannins during ensiling holds key both at ensiling and at rumen level to check N degradation and decrease its excretion.

Plant phenolic compounds and flavonoids are the largest and best-studied natural phenols that possess a series of biological properties and act on biological systems, such as antioxidants, antimicrobials and immunostimulants, which in turn, are associated with a reduction in the incidence of various human diseases. The shift in research to feedstuff endogenous factors, which influence proteolysis and lipolysis, may have a significant contribution on ruminant products (meat, milk) and their transmission to human food chain. Any qualitative variation in ruminant food products with naturally rich conjugated linoleic/linolenic acid (CLA) and other ω3 fatty acids (FA) can thus be influenced by animal's diet. Ruminal biohydrogenation is heavily influenced by PSM, which includes polyphenol oxidase (PPO), FA oxidation and tannins, and the effect is a complex set of mechanisms directly affected by PPO or indirectly by passage rate, lipid encapsulation, shift in the ruminal microbial population,

modulation of protein and fiber degradation and interactive effect of other PSM [16]. The animal performance with silages from PSM-rich forages attributed not only to differences in their nutritive value but also to interactive effects impacting differently on feeding motivation and digestive efficiency [17].

Usefulness:

- **Reducing nutrient drainage**: Decrease in protein and N compound degradation during ensiling and rumen fermentation.
- **Improving nutrient use efficiency and enhancing P:E ratio**: Efficient use of N in grasses by ruminants decreases its excretion, reduces requirements in diet and increased net return per cow.
- **Protecting environment**: Reduces environmental pollution thereby promoting environment-friendly livestock production.
- **Improving livestock products quality**: Alters ruminal biohydrogenation process and improves FA profile of milk and meat.

Possible outcome: Research outcome will help identify tannins and other phytochemicals which can be used as silage additives. Tannins shift N excretion from urine to feces and from soluble to insoluble N forms in feces. This undigested form of N from plant residues mineralizes more slowly than microbial and endogenous N, and these shifts in N forms could reduce ammonia and nitrate losses from ruminant production system and thus contribute to reducing the protein and NPN supplements in the animal feed. Besides improving N usage and aerobic stability of silages, the ingested phytochemicals also have significant role on improving ruminant livestock products and their shelf life [18]. This will augment "Green Consumerism" and the naturally improved products could be placed on the market at higher prices with the brand name of "environment-friendly products." Ultimately, farmers will feel encouraged to adopt bioactive forage-enriched feed for livestock feeding.

3.3.2. Use of phytochemical-rich plants and nutrient usage

The use of phytochemicals in ruminants can induce beneficial effects, most-importantly, the role of condensed tannins on retention of dietary N (reduced urinary output) and its overall usage vis-à-vis efficiency of energy utilization. Ensiling of N-rich forages (e.g. alfalfa/lucerne, berseem, cowpea, etc.) transformed majority of protein N into non-protein N (NPN), which can be inhibited to some degree by accelerating the rate of pH decline during silage fermentation, but compared to cereal forages, structural difference in leaf: stem ratio and its physicochemical characteristics, low WSC content and high buffering capacity make it difficult to ensile resulting in proteolysis. Forages containing condensed tannins (CT) undergo less proteolysis during ensiling, and transformation of their plant protein N into NPN is inhibited compared to forages without CT [10, 19, 20]. Therefore, adding tannins during ensiling holds key both at ensiling and at ruminal level to check N degradation and decrease its excretion to the environment. High level of tannins may adversely affect the activities of silage bacteria [21]. Co-ensiling

sainfoin and alfalfa improves fermentation in silos and increases total tract digestibility, suggesting positive associative effects of the two forages [20]. The optimal ensiling and ruminal fermentation for alfalfa and sainfoin were observed at approximately 60:40 ratio (DM basis). It also reduces proteolysis and preserves the nutritive value with sainfoin relative to alfalfa alone. Both total phenol and total tannin contents contributed to the decrease in lactic acid production. Fasuyi et al. [22] found 4% molasses and 14 to 21 days ensiling period optimum and most suitable for effective ensiling of *Tithonia diversifolia* leaves. They also observed a gradual decrease in major anti-nutrients (phytin, tannins, oxalates, alkaloids, flavonoids) with lengthening duration of ensiling. However, there are reports that tannins suppress the production of lactic acid during ensilage [23]. A number of additives that include chemical inhibitors, such as acids, formaldehydes, and various salts, and biological stimulants (LAB and other bacteria) expedite lactic acid production to support ensiling process [24]. The resistance mechanisms of *L. plantarum* include the ability to degrade phenolic compounds [25] such as tannin, by the action of novel tannin acylhydrolase [26] and gallate decarboxylase enzymes [27].

3.4. Concept of mixed silage

The concept of mixed silage has widen the scope of incorporating grains, protein concentrates, leguminous forage crops, tree forages and other conventional and unconventional tanniniferous forage crops with the conventional one for silage making. Making of mixed silage involving seasonal availability of feed resources allows the farmers to opt for a variety of forages, for example monsoon herbages, tree forages and browses, including that of conventional grasses and cultivated fodder. It also widens the scope of incorporating non-conventional fodder resources like cactus, thorny non-toxic plants/weeds and phytochemical-rich plant resources. Corn and legume silages are commonly fed together in rations for dairy cattle, one complementing the other for the deficit N and energy sources, respectively. Thus, the fermentable carbohydrates in corn silage may complement the rumen degradable N (RDN) in legume silage, which may decrease ruminal N losses. Above all, the N intake affects the amount of N excreted via manure, whereas types of carbohydrate (starch in corn silage vs. sugars in grass silage) and forage species (legume vs. grasses) have greater impacts on the route (fecal or urinary) of excretion. Besides agronomic benefits of grass-legume mixtures, there are positive associative effects contributing to voluntary intake due to a greater motivation of animals to eat mixtures along with decreased urinary N excretion and increased N retention [28]. Thirumurugan et al. [29] evaluated cereal-legume mixed silage to combat feed and water scarcity and sustaining production during hot summer in semi-arid regions of India. Total mixed ration (TMR) silage is a way forward in this direction.

3.4.1. Why mixed silage?

Monotonous use of a single fodder (e.g. maize) in silage making limits the farmers to adopt the technologies, particularly in unfavorable geographical and climatic regions (semi-arid and arid regions). Therefore, combination of grasses and legumes is an alternative solution to the success of the ensiling process. The purpose of the addition of legumes to silage is to supply N/protein for microbial protein synthesis, reduce protein degradation in the rumen and

increase amino acid absorption in the intestinal tract. The combination of grasses and legumes in ruminant feed is very effective for a highly nutritious diet. This allows the farmers to use its surplus or seasonal plenty or available at hand plant biomass resources to preserve as silage to feed during scarce or unavailability. Successful ensiling can be evaluated by determining the relationships between fermentation characteristics and microbial diversity in silages. Reducing the moisture content of the crops through substitution with other high DM forages could be another approach. Moreover, nutrient composition analysis following up with palatability study can very well evaluate the combinations of different silages involving local grasses, tree forages, browses and monsoon-favored less/non-tested abundantly grown plant biomass. This versatility allows the farmers to use their wisdom to choose and harvest the available forage biomass at hand for preserving as silage. Above all, year-round feed and nutrient supply to the livestock can certainly enhance per animal/whole farm productivity, thereby enabling the livestock husbandry sustainable and profitable. Some of the questions that continue to be answered for harnessing possible beneficial effects of plants rich in phytochemicals as a part of silage are

- Can mixed silage concept is more versatile, if at all, the effect of high N containing feedstuffs on silage quality and preservation process?
- Tannins/polyphenols that are suitable for adding during ensiling need to be identified based on their chemical characteristics, affinity to form complexes during ensiling and rumen fermentation and release of tannin-protein complexes at different pH of stomach and intestine, and also keeping in view their toxic and anti-nutritional effects.
- Comparing results from simulated rumen *in vitro* system studies for stability of complexes and release of bound N from rumen of silage with tannins added during ensiling, can help evaluate efficacy of tannins for utilization of N in grasses and leguminous forages by ruminants.
- Feeding trials in different ruminant species fed on grass silages with or without tannins and the effects of supplemental tannins pre- and post-feeding on excretion of N and N-metabolites for evaluating efficacy of tannins on overall N economy.
- Effect of other plant phytochemicals on ensiling process and post-consumption effect on ruminal N and energy use efficiency both in vitro and in vivo.

3.4.2. Competing conventional silage

The plant, microbial and environmental factors that influence the fermentation process determine the nutrient value of the mixed silage. These factors must be considered as an integrated package to facilitate optimum forage preservation process. Moreover, it is now observed that the use of ensiled alternative forages have positive influence on voluntary feed intake (VFI), nutrient use efficiency and productivity of livestock systems [2, 30]. This would encourage the livestock farmers to preserve nutrients for future use and sustain whole farm productivity. A list of mixed silage evaluated in various countries in the feeding of native ruminant livestock is detailed in **Table 1**.

Silage types			DM	OM	CP	NDF	ADF	Citation
Local name	**Region/Country**	**Botanical name***						
Cholai* (100)	India	Amaranthus sps	22.8	87.2	8.80	55.5	49.6	[30]
Bajra* (100)	India	Pennisetum typhoides	36.9	87.8	8.18	65.8	46.7	
Cholai + Bajra (50:50)	India	–	28.6	87.5	8.54	60.3	47.8	
Cholai + Cenchrus* (50:50)	India	Cenchrus sps	26.9	86.2	7.32	55.2	41.5	
Jojhru* + Cholai (50:50)	India	*Crotalaria medicaginea*	30.6	87.4	10.28	52.2	38.5	
Jojhru + Cenchrus (50:50)	India	–	27.8	87.0	8.76	58.8	42.5	[2]
Jojhru + Bajra (50:50)	India	–	32.7	87.0	9.88	59.2	41.5	
Cactus* + Ardu[1,*] (80:20)	India	*Opuntia* sps + *Ailanthus excelsa*	25.8	85.1	8.70	51.7	35.0	[30, 31]
Cactus + Gram straw* (80:20)	India	*Cicer arictinum*	28.6	82.9	8.36	54.5	38.6	
Cholai + Moringa[1,*] (80:20)	India	*Moringa oleifera*	32.2	86.1	12.22	52.5	34.2	[2]
Moringa + Bajra[1] (70:30)	India	–	37.2	88.3	12.56	55.4	38.2	
Cholai + Ardu[1] (80:20)	India	–	30.8	86.8	9.28	53.8	41.5	
Oat* + Ardu[1] (75:25)	India	*Avena sativa*	35.8	88.2	8.22	58.6	44.8	
Barley* + Ardu[1] (75:25)	India	*Hordeum vulgare*	36.2	87.9	8.02	59.5	44.2	
Oat + Lucerne* (75:25)	India	*Medicago sativa*	32.6	87.3	10.51	52.8	39.5	[29]
Oat + Lucerne + Ardu[1] (75:12.5:12.5)	India	–	36.0	87.8	10.24	53.6	39.2	
Cactus + Acacia* (67:37)	Zimbabwe	*Acacia angusitissima*	38.0	81.6	25.0	63.4	57.4	[32]
Cactus + Leucaena* (67:37)	Zimbabwe	*Leucaena leucocephala*	44.0	82.4	20.0	57.3	52.3	
Cactus + Calliandra* (67:37)	Zimbabwe	*Calliandra calothyrsus*	41.0	84.1	21.9	72.2	55.4	
Cactus + Siratro* (67:37)	Zimbabwe	*Macroptilium atropurpureum*	42.0	85.3	12.5	65.7	58.8	
Pennisetum* (100)	Indonesia	*Pennisetum purpureum*	31.2	87.5	5.6	66.6	34.4	[23]
Pennisetum + Calliandra (75:25)	Indonesia	–	33.6	89.7	10.6	59.7	25.4	
Pennisetum + Calliandra (50:50)	Indonesia	–	37.0	90.9	14.2	56.0	26.1	
Pennisetum + Calliandra (25:75)	Indonesia	–	40.7	92.4	17.3	54.4	17.5	
Calliandra (100)	Indonesia	–	46.5	93.4	20.2	53.8	13.4	

Silage types			DM	OM	CP	NDF	ADF	Citation
Local name	**Region/Country**	**Botanical name***						
Aruana grass* (100)	Brazil	*Panicum maximum*	28.2	–	8.95	67.1	41.3	[33]
Aruana grass + Gliricidia (75:25)	Brazil	–	28.3	–	10.07	60.9	40.1	
Aruana grass + Gliricidia (50:50)	Brazil	–	28.1	–	11.06	53.7	37.2	
Aruana grass + Gliricidia (25:75)	Brazil	–	27.5	–	12.0	46.5	30.4	
Gliricidia* (100)	Brazil	*Gliricidia sepium*	27.6	–	12.83	39.5	25.6	
Gliricidia (100)	Vietnam	–	21.7	92.9	20.3	52.7	35.4	[34]
Mexican sunflower	Nigeria	*Tithonia diversifolia*	17.6	–	17.00	–	–	[22]
Moringa (100)	Nigeria	–	31.9	97.9	18.45	11.2	11.1	[35]
Moringa + Wheat* offal (50:50)	Nigeria	–	36.2	90.9	14.35	12.1	9.95	
Moringa + Guinea* grass (50:50)	Nigeria	*Panicum maximum*	32.1	97.9	8.25	12.3	11.1	
Moringa + Guinea* grass + Wheat offal (50:10:40)	Nigeria	–	32.1	97.5	10.48	11.2	9.16	
Moringa + Guinea* grass + Wheat offal (50:20:30)	Nigeria	–	32.3	97.6	12.27	11.0	9.16	
Moringa + Guinea* grass + Wheat offal (50:30:20)	Nigeria	–	33.5	97.5	13.13	10.9	8.90	
Moringa + Guinea* grass + Wheat offal (50:40:10)	Nigeria	–	35.9	97.6	13.40	10.7	8.54	
Amaranth	Iran	*A. hypochondriacus*	48.8	91.8	14.70	30.0	17.2	[36]
Fruit* byproduct silage	Greece	*Punica granatum*	29.2	95.9	12.00	21.8	16.9	[37]
Cassava	Vietnam	*Manihot esculenta*	26.7	92.8	21.70	51.4	37.2	[34]
Cassava leaf	Indonesia	–	30.7	92.9	16.20			[38]
Cassava leaf	Nigeria	–	30.4	97.8	15.46	–	–	[39]
Cassava peel	Nigeria	–	29.2	97.8	5.72	–	–	

*Forages used in silage display botanical names.

[1]Forages in dry form.

DM, dry matter; OM, organic matter; CP, crude protein; EE, ether extract; TCHO, total carbohydrates; NDF, neutral detergent fiber and ADF, acid detergent fiber.

Table 1. Dry matter and nutrient composition (% DM basis) of different types of unconventional silages.

3.5. Silage from alternate forages

3.5.1. Amaranthus silage

Amaranth is a dicotyledonous species and commonly considered as a pseudo-cereal, which has a good yield performance up to 86.4 t fresh forage/ha [40] with promising nutritive value [30, 41–43] and CP up to 285 g/kg DM with useful minerals including Ca, Fe, Zn, Mg and P. It is adaptable to varying climatic and agronomic conditions, tolerance to drought as well as a low water requirement [44]. The use of Amaranthus silage in the diet of fattening Moghani lambs up to 300 g/kg of dietary DM improved total gain and carcass weight without any adverse effect on lean-to-fat ratio and animal health and demonstrated its replacement value for maize silage [36]. A small bag ensiling technology is being promoted as a useful and low cost tool to improve production in smallholder livestock farms [45].

3.5.2. Moringa silage

Moringa oleifera has attracted the attention of researchers in recent times, and its intensive cultivation with the application of fertilizer and water supply gives a DM yield up to 120 tonnes/ha, with 7–8 cuttings in a year [46]. Moringa leaves are high in CP and phytochemicals that reported to have a positive influence on lactation performance [47, 48]. Sole Moringa silage, or in combination with fresh *Panicum maximum* in equal proportion, may not be promising dry season feed conservation strategies for ruminants, while silage mixtures of 50% Moringa +10–30% Guinea grass and 20–40% wheat offals showed great potentials [35]. It should preferably be ensiled in mixtures with conventional and/or unconventional forages to increase the VFI and nutritive value of the silage. This is often considered as a perennial forage surplus to preserve as silage to meet round the year feed requirements.

3.5.3. Cactus silage

Cactus, particularly the *Opuntia* species, is grown in semi-arid regions of many tropical countries and is often fed to livestock during summer to provide both feed and water [31]. However, the excess biomass during other season can be preserved suitable as silage for feeding during scarcity [30]. It was observed that mixing cactus and browse in silage making improved both DM and N content in the product. Similarly, it can be mixed with legume forages and hays by supplying a degradable source of organic matter. The cactus + browse or cactus + legume silages improve microbial protein flow to the lower gut for digestion and supply of amino acid for maintenance, growth and production. These silages could be used in livestock feeding to improve livelihoods in drier and resource constrained farming communities by providing opportunities for conservation of forage and maintaining their animals in periods of feed scarcity. The nutritive value of silage from cactus cladodes was evaluated and found acceptable quality silage based on pH, organic acids contents and voluntary intake. It might be advantageous to ensile cactus mixed with other ingredients and improving utilization of poor quality roughages with the addition of cactus-browse silages as supplements [31]. Abidi et al. [49] ensiled fresh cactus cladodes with olive cake and wheat bran and found no adverse effect on

digestible nutrient intakes by replacing with oaten hay. In addition to feed shortage, water scarcity compromises livestock performances in dry areas. Because of its succulence, cactus could overcome this constraint as ruminants do not need to drink water when receiving cactus cladodes (35 g DM/kgW$^{0.75}$) [50]. It is reported that ensiled mixture of spineless cactus, olive cake and wheat bran could be used to replace totally or partially oaten hay without affecting lamb performances and meat quality [49]. It is thus advocated to go for mixed silage with cactus and protein-rich dry forages (e.g. Ardu leaves, gram straw, pea crop residues), so as to balance the minimum moisture content (i.e. 35–40%) in making of good quality silage [31]. A reasonable intake of 3–4 kg cactus silage in adult sheep was recorded that meet 900–1200 g DM and enough nutrients to support minimum production during scarcity.

3.6. Silage from by-products

3.6.1. Silage from fruit and vegetable co-products

Utilization of fruit and vegetable co-products, such as grape, tomato, olive or citrus pomace that are voluminously produced and have an important impact on the environment, in the animal feed holds promise in expanding the forage biomass, thereby meeting the increasing demands of feeds and fodder. Besides the fact that fruit and vegetable co-products are good sources of phenolic constituents [12, 51] that act as natural antioxidants, and research emphasis has now been directed at use of these co-products in improving products' quality [14]. They evaluated pomegranate byproduct silage supplementation to growing lambs and found improved nutritional and functional qualities as indicated by the increase in essential FA, intramuscular fat, total phenolic content and antioxidant activity.

3.6.2. Pineapple fruit residue silage

National Institute of Animal Nutrition and Physiology (NIANP), Bengaluru (India), has developed a silage technology to preserve pineapple fruit residue (PFR) and use it as fodder for livestock [52]. More than 70% of pineapple fruit is wasted during processing in industry, and there is potential availability of PFR to the tune of 1.35 million tonnes per year in India. PFR is high in moisture and sugars and thus can suitably preserved in the form of silage, which otherwise become a waste in processing industries. Nutritionally, PFR silage is demonstrated as a valuable fodder resource comparable to maize green fodder and increases milk yield and quality.

3.6.3. Cassava silage

Cassava (*Manihot esculenta*) is known as a highly productive tropical crop that is traditionally cultivated to produce roots for human consumption or for starch production. The yield of cassava leaves is recorded as much as 4.6 thousand tonnes of DM/ha when taken as a by-product at root harvesting. Cassava leaf silage was successfully introduced to small holder farmers in Indonesia [38]. The chemical and microbiological composition, silage preparation and the effects of LAB inoculants on silage fermentation of cassava residues including cassava leaves, peel and pulp were studied to effectively use in livestock diets [53, 54]. They found improved fermentation quality with lower pH, butyric acid and higher lactic acid when the residues were ensiled with LAB inoculants.

3.7. Ensiling on nutrient composition and utilization

Differences in WSC and protein (particularly, the A and B fractions) contents and fermentative characteristics between plant parts and plant species contribute to the differences in ensiling process, be it lactic acid production, pH reduction and modification/reduction in the phytochemical constituents. The use of molasses has been in practice for stepping up the initial fermentation process during ensiling. It is suggested that a critical WSC concentration in herbage for successful preservation as silage without additives is 30 g/kg DM. Sugars, such as fructosans, starch, pectins and soluble fiber content, greatly decline during the fermentation process [55]. A part of the OM gets lost in the initial phase owing to respiration of plants and during fermentation to CO_2 and other fermentation products and storage of silage by microbial activity. Total DM losses for optimal lactic acid fermentation are relatively low and should range between 2 and 6%. The proteolytic activities are restricted when the pH of the silage is ≤4.3 [56], and in good silage, the process will stop earlier and limit the loss of protein. Tannin might limit the proteolytic activities and reduce the loss of silage CP (soluble NPN) [19]. Different ratios of grass to red clover silage in TMR demonstrated improved performance when they were offered as a mixture than when fed alone [57]. Red clover contains PPO, which binds protein and it tended to reduce whole body N balance at higher inclusion levels due to increased partitioning of N into urine and feces. Legumes that contain CT also have the potential to reduce the degradation of plant protein to NH_3-N in the rumen, thereby releasing proteins in the abomasum, leading to improvements in feed efficiency and reduction of N excretion. Research emphasis should therefore be needed to explore the interactions of CT-containing legume feeds with other dietary components, fiber digestion and the consequential N partitioning effects, thereby reducing N excretion and improving efficiency and environmental quality.

3.8. Ensiling effect on phytochemicals/anti-nutritive factors

Phytochemical determination showed that ensiling reduces the presence of some anti-nutritional factors such as tannins, phytic acid and trypsin inhibitors [58]. A low pH, which is critical to make good silages from wet crops, also dissociates tannin-protein complexes and may compromise formation of rumen escape protein that can improve protein utilization. Invariably, ensiling of tannin-rich, legume, cereal or mixed forages shows a pH decline not beyond 4.0, and hence, any possibility of dissociation of tannin-protein binding complex does not arise, which requires pH of 2–3 [59]. Increase in ensiling duration also reduces tannin concentration. At pH range 3.5–5.5, insoluble tannin and plant leaf protein complex was established [60]. A reduction of 25 and 42% in the tannin content of fresh cassava and Gliricidia tops, respectively, was found after ensiling [34]. This phenomenon can be correlated to hydrolysis of hydrolysable tannins. Moreover, diets containing 2–4% of CT reduce proteolysis during ensiling and rumen fermentation by up to 50% [61]. Similarly, a continuous decline in HCN to the tune of 68 and 43 % was found in ensiled cassava and Gliricidia tops, respectively after 2 months of ensiling [60]. Handling and ensiling process and the initial environment of the aerobic phase created favorable environment for reducing the HCN. A rapid reduction in pH restricts the enzyme activities that reduce the speed of HCN elimination during storage. Pyrrolizidine alkaloids remain unaltered in silage and are not toxic [62]. The PPO activity, associated predominately with the detrimental effect of browning fruit and vegetables,

showed potential to inhibit proteolysis that draws interest to improve animal forage quality through greater N utilization [63]. The mechanism for PPO protection of plant protein in the rumen is a consequence of complexing plant protein, rather than protease deactivation, and these complexed proteins reduce protein digestibility in the rumen and subsequently increase undegraded dietary protein flow to the intestine. It catalyzes the conversion of phenols to quinones, which are extremely reactive and bind with cellular nucleophiles such as proteins to form protein-bound phenols. Red clover and cocks foot (*Dactylis glomerata*) showed high PPO activity compared to other forages [64]. There are several reports on the positive effect of PPO on preserving polyunsaturated FA (PUFA) within rumen simulation models [17] and limiting post-harvest proteolysis [64, 65]. The effect of PPO on inhibition of ruminal proteolysis and biohydrogenation is reported at 25 and 11%, respectively [66]. Thus, the benefit of red clover silage is attributed to a reduction in lipolysis in silo and an increase in beneficial C18 PUFA in animal products. A number of factors, e.g. cultivar, growing season, stage of maturity, sward management including forage conservation method and cell damage, play a role in the extent of enzyme activity.

3.9. Total mixed ration (TMR) silage

Ensiling TMR allows preservation and saves labor at the farm as it saves daily preparation cost of TMR with succulent fodder. This concept of silage making is aimed at ascertaining nutritional adequacy to livestock for maintenance and/or production. Production of TMR silage and feeding to production stock in a livestock farm are gaining rapid acceptability. However, the fermentation process of the substrates during ensiling may influence various nutritional components, and therefore, the loss should be kept minimum for easy adoption and maximize nutrient utilization efficiency. Balancing CP and total carbohydrates content with respect to concentration of fermentable N and sugars would provide a desired reducing environment for anaerobic degradation to lactic acid and rapid drop in pH. Brewer's grains are found to be a suitable by-product for ensiling as it improved aerobic stability when ensiled with various feeds as a TMR [67]. Five different silage types with cassava by-products (peel and pulp at different ratio) (40%) with corn husk (42%), Brewer's grain (14%) and molasses (3%) were evaluated and recommended as a useful energy source in Thailand during the dry period [54].

3.10. Aerobic stability of silage

It is a key factor in ensuring that silage provides well-preserved nutrients to the animal with minimal amounts of mold spores and toxins. When silage is exposed to air on opening the silo, fermentation acids and other substrates are oxidized by aerobic bacteria, yeasts and molds, and the stability is thus dependent principally on following factors [68]:

i. **Biochemical and microbiological factors**: Development of yeasts and molds during plant growth and during field wilting or storage and concentration of undissociated acetic acid in silage.

ii. **Physical and management factors**: Silage density and porosity are key physical factors that affect the rate of ingress of O_2 into the silage mass during the feed-out period. A target for potential silage aerobic stability is generally 7 days including the time in the feed

trough. Speed of harvest needs to be coordinated with packing to achieve a minimum silage density of 210 kg DM/m^3 by the time of feed out and a rate of silage removal to match or exceed the depth of air penetration into the silo.

iii. **Type of additives**: Use of additives is advisable when there is risk of meeting these objectives.

iv. **Silo sealing**: Post-exposure sealing of silage pit/bags/drums helps prevent aerobic exposure and infestation of bacteria, molds and yeast/fungal growth.

3.11. Spoilage and fungal contamination

The epiphytic microbial populations are the starter culture and the survival and activity of these populations are also among the factors influenced by the characteristics of the crop at the time of ensiling and often contribute to spoilage and could be a potential health risk. Therefore, the types of LAB rather than the total numbers of bacteria present in the epiphytic populations may be more important in determining the efficiency of the fermentation process. Manuring onto forage prior to ensiling increases numbers of epiphytic enterobacteria (e.g. *Bacillus* and *Clostridium* spp.) and contact of the forage with soil can also increase yeast and mold counts in the silage [69]. Although these microorganisms are usually inactivated during ensiling, they can become active and contribute to accelerated spoilage when the silage is exposed to air upon feeding. Well-preserved silages are dependent on appropriate fermentation after storage, which results in low pH and production of sufficient acid to inhibit the growth of undesirable microorganisms. Lactate-oxidizing yeasts are generally responsible for the initiation of aerobic spoilage, and the secondary aerobic spoilage flora consists of molds, bacilli, listeria and enterobacteria [70]. The activity of harmful microorganisms not only reduces the silage quality (e.g. formation of butyric acid) but also increases the losses of energy and DM [71]. Hexoses are fermented to carbon dioxide and hydrogen, and subsequent decarboxylation and deamination of amino acids by these bacteria contribute to a decrease in the quality and quantity of forage. Yeasts also ferment sugars to ethanol and carbon dioxide with higher fermentation losses. Spoilage after opening the silage pit or bags seems to be concurrent problems faced by many farmers because LAB typically reduces the concentration of acetate, which is strongly antifungal, and increases concentration of lactate, which is a growth substrate for spoilage yeasts [6]. When silage is exposed to air during storage or at feeding, aerobic spoilage leads to increase in pH and losses of DM and nutrients [72] due to lactic acid degradation by mainly the lactic acid-utilizing yeasts (e.g. *Pichia*, *Candida*) [73]. Difference in anaerobic degradation of cereal and legume forages during silage making leaves more residual WSC than do legume silages, which is a readily available source of energy for the animal. But, upon exposure to air, these WSC are readily utilized by spoilage microorganisms and often become more prone to aerobic deterioration than legume silage.

Growth of spoilage fungi in baled silage is not of random occurrence but is facilitated where in-bale environments allow the fungi to survive, colonize and reproduce. Visible fungal growth was observed on baled grass silage during the winter feeding season [74]. The factors analyzed are the concentrations of ethanol and lactic acid, DM content, bale tying, month of bale feed-out, age of bales, polythene film damage, ryegrass dominance, bale storage location

and volatile FA concentrations. Besides, the environmental factors inside the silo, e.g. moisture content, pH, acid and gas composition, are likely to influence the species composition of the fungal population and the extent to which mold colonization occurs. Oxygen ingress causes excessive moisture or dryness, condensation, heating, leakage of rainwater and insect infestation of the silo, leading to undesirable growth of microaerobic acid-tolerant fungi, which may lead to mycotoxins production in this substrate [75]. Mycotoxin-producing molds, Bacillus sps and *Listeria monocytogenes* in aerobically deteriorated silage form a serious risk to the quality and safety of milk and to animal health. An average of 32% positive cases observed with most frequent fungal species from *Aspergillus, Penicillium* and *Fusarium* in pre- and post-fermented sorghum silage samples [76]. Thus, periodic monitoring is essential to prevent the occurrence of mycotoxicosis particularly in countries with hot and humid climates.

3.11.1. Controlling spoilage

Addition of high levels of propionic acid is effective against aerobic spoilage, but its use has been restricted because of its corrosive nature, relative expensiveness, involvement in VFI depression and variable sensitivities of yeast [77]. Control of silage fermentation by microorganisms seems to be a safe and inexpensive alternative, and in this line, LAB inoculation has been recommended to improve the aerobic stability of silage [8]. Killer yeasts (e.g. *Kluyveromyces lactis*) are known to secrete a killer protein that is lethal to specific yeasts (e.g. *Saccharomyces cerevisiae*) in a model of silage fermentation [78]. Genetically modified killer strain of *K. lactis* constructed to avoid dependence on substrates of lactose/lactic acid, a principal product of silage fermentation, which reduced aerobic spoilage.

3.11.2. Microbial inoculants

The mechanisms by which the inoculants alter silage fermentation and potentially improve animal performance are numerous. It supports accelerated post-ensiling decline in pH enabling quality silage production, improves stability and DM preservation, conserves nutrients, enhances aerobic stability and improves voluntary intake, nutrient utilization and efficiency of production. There may be increase in lactic/acetic acid ratio and reduction in proteolysis and protein deamination, thereby allowing better utilization of WSC and increase in DM recovery [79]. The problem of aerobic instability could be prevented by the use of microbial inoculant *L. buchneri*, a heterofermentative LAB, which could improve the aerobic stability of silages through the production of acetic acid from lactic acid during the anaerobic phase of silage conservation [71, 80, 81]. The natural populations of LAB in fresh crops are often heterofermentative and low in number, and thus homofermentative bacteria are used as inoculants to improve silage preservation. This accelerates the initial phase of the fermentation process via anaerobic degradation of WSC into lactic acid with a rapid decrease in pH and thereby preventing growth of spoilage microbes, molds and other contaminants and supporting preservation and storage without further deterioration in quality. Usually, selected homofermentative LAB have been used to improve the efficiency of the fermentation process to minimize DM and nutrient losses over conservation [82]. To prevent the aerobic deterioration of silage, heterofermentative LAB species, such as *L. brevis* and *L. buchneri*, have been developed as silage

additives [6, 83–85]. Dual-purpose inoculants containing both homo and heterofermentative LAB have recently been developed and the beneficial effects on aerobic stability have been reported [86]. Some isolates of *L. buchneri*, besides producing acetic acid can produce ferulate-esterase enzyme, which hydrolyses feruloyl ester linkages between lignin and hemicellulose, and thus advocated to potentially improve fiber digestibility of forages during ensiling [87]. The diversity of LAB species inhabiting silages stabilizes its fermentation quality and supports preservation. LAB inoculants (viz. *L. plantarum*, *L. rhamnosus*, *L. acidophilus*, *Pediococcus acidilactici*, *P. pentosaceus*, *Enterococcus faecium*, *Lactococcus lactis*, etc.) are safe and easy to apply, non-corrosive, do not pollute the environment and are regarded as natural products.

3.12. Therapeutic silage

Phytochemicals have antimicrobial properties against *E. coli*. The use of a high-quality PSM-containing forage may have the dual benefit of being a good-quality forage and reducing *E. coli* shedding [88]. Significant potential to use plants rich in bioactive compounds (saponin and tannin) for enhancing animal health and productivity that include reproductive efficiency, milk and meat quality improvement, foam production/bloat control, methane production [89] and Nematode control [90] has now been realized. The physicochemical structure and concentration of the phenolic compounds in the diet modulate the beneficial effects, and therefore, conceptualization of producing "therapeutic silage" involving forages rich in desired bioactive components would harness clinical and health benefits, besides modifying the yield and quality of meat and milk.

4. Protecting environment: green livestock production

Rapid breakdown of herbage proteins in the rumen and inefficient incorporation of herbage N by the rumen microbial population are major determinants of N (and C) loss and pollution in pasture-based livestock production system. Thus, when livestock are given fresh forages, they can waste 25–40% of the forage protein-N during ruminal fermentation. An increase from 23 to 34% in rumen N use efficiency through feeding higher WSC containing grasses could result in a 30% reduction in N_2O and NH_3 emissions [91]. Similarly, increasing the digestibility of cell walls in forages has been practiced to lower CH_4 losses, but in fresh grass and grass silage, the scope of this approach seems limited. CH_4 production in ruminants tends to increase with maturity of forage fed, and CH_4 yield from the ruminal fermentation of legume forages is generally lower than the yield from grass forages [92]. Shifting the animals from grass to legume plant species tends to decrease the enteric emission due to lower proportion structural carbohydrates and faster rate of passage which shifts the fermentation pattern towards higher propionate production. Further, enhancing N use efficiency in the rumen may also contribute to a reduction in the amount of C (both as CO_2 and CH_4) excreted. The concept of mixed or TMR silage may certainly address these concerns and enable eco-friendly livestock production. The impact of the form of C relative to N and the effect at different C:N ratios in terms of rumen function and conversion efficiency is an area of considerable promise that requires further detailed research.

4.1. Eco-friendly silage

The use of home-grown protein-rich feeds (e.g. forage legumes) with multiple positive effects associated with their role in N_2-fixation and lower protein degradation emanated from tannin-protein interaction, thus contributing to nutrition and the environment. The PSM in forage silage can have positive effects on animal nutrition in terms of (i) improved N utilization; (ii) animal health (e.g. tannin-parasite interaction) and (iii) the environment through reduction of CH_4 and N emission. Enhanced in vitro DM digestibility and low methane production observed in vegetable residue silage inoculated with *L. plantarum* [92]. Inclusion of red clover in silages is found to be a promising strategy to bring in combined effect of improved animal performance with reduced environmental pressure [17] due to the presence of active POP in chaffed forages that act on exposed plant cell contents [63]. There is thus a need to go for selective plant breeding to develop tropical forages with decreased plant fiber and lignin content, increased WSC, increased content of S-amino acids, desired phytochemicals, etc.

4.2. Managing silage effluent

In some intensive agricultural areas, silage effluent may be one of the commonest forms of agricultural pollution contaminating water bodies. Sealing of silos with cement or lining with plastic sheets, and use of plastic bags/drums preserves the leachate that usually contains high amounts of nitrates. Harvesting forage for silage making at the correct moisture content and proper storage will reduce the volume of leachate from the silo. Silos and trenches should be located away from wells/water bodies to reduce the possibility of effluent polluting groundwater sources. A vegetated area between the trenches will be of greater usage to utilize the N-rich leachate or it can be applied to land as a source of crop nutrients.

5. Forage banking and meeting feed scarcity

Preservation of forage as hay and silage is intended at banking during surplus to meet the scarcity during unavailability or natural calamities. In other words, these technologies would evenly and adequately supply the bulk of feeding to livestock, thereby insulating any drop in production. Feeding hay or silage to livestock helps reduce the amount of concentrate feeding and thereby the cost of feeding. The concept of haylage, mixed silage and TMR silage has widen the scope of feed banking and nutritional optimization for higher productivity. Silage making is not only a process of feed preservation but it also preserve nutrients, phytogenic substances, succulence and completeness of a ration, thereby further the scope of feed and nutrient banking.

6. Conclusion

Seasonal feed scarcity is a concurrent problem that farmers usually face besides natural calamities like drought, flood, cyclone, earthquake, etc., which has a significant impact on sustaining animal agriculture and guaranteeing profitability. Ensiling of surplus forage biomass will

ensure round-the-year feed supply and safeguard production decline in times of feed scarcity and also could able to make ready the animal for the next production year, thereby enhancing per animal productivity/whole farm output. Some of the inherent problems associated with ensiling are decline in the feeding value due to protein and amino acid breakdown and concomitant accumulation of ammonia. Assessment of the likely importance of microbial inocula and enzyme additives for stimulating various stages of ensiling process (e.g. separation of lingo-cellulose), likely impact of microbial origin formic acid vs. petrochemical sources and interactive function of microbial communities in ensilage are some of the areas of concurrent and ongoing research. Newer research areas include silage with herbal additives, phytochemical-rich plant biomass, therapeutic silage that promises veterinary health care (e.g. parasite control, control of bloat, acidosis), antioxidant-rich silage, high-moisture silage, etc. There is always animal and human health concern pertaining to consumption of deteriorated silages due to secondary aerobic spoilage by molds, bacilli, listeria and enterobacteria. Novel microbial approaches to solving the problem of aerobic deterioration during the feed-out period are needed. Silage inoculants can facilitate the ensiling process, but they can never be a substitute to the fundamental factors (plant maturity, DM content, oxygen exclusion) that are keys to making good quality silage. Utilization of agroindustrial by-products/co-products, including fruit and vegetable processing co-products, can be effectively used in mixed silage or TMR silage, which seems to be an underexploited source of dietary supplementation to farm animals with functional compounds and the production of value-added products. A challenge in the future is to complete studies on plant lipid fractions in conjunction with PSM and PPO in order to discriminate between effects of plant lipids on FA biohydrogenate intermediates. This may become the basis for achieving more sustainable, less expansive and healthier ruminant-derived human food.

Acknowledgements

The author is thankful to NICRA and CSWRI under ICAR, New Delhi, for providing the facilities to undertake the work on silage.

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Chapter 3

Effect of Bioregion on the Size and Production Efficiency of Bonsmara Cattle in Semi-Arid Parts of Southern Africa

Edward C. Webb, Pieter C. Visagie and
Japie van der Westhuizen

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.72713

Abstract

The effects of bioregions in the semi-arid parts of Southern Africa on growth, size and reproduction efficiency of Bonsmara cows are investigated. The regional distribution of cattle influences the growth, size and reproduction efficiency of cows, and provides evidence for an optimal cow size for different bioregions. Effects of bioregion on growth and reproduction of extensive beef cattle is complex, because the proportion of variation in growth traits explained by bioregion, depends on the physiological stages of growth, e.g., birth, weaning, 12- or 18-month growth stages. For production efficiency, weaning- and yearling weights as well as age at first calving (AFC) and reproduction index (RI) were influenced most by bioregion. Management practices, such as livestock recording and improvement strategies, and better nutrition at weaning and yearling age, limit the negative effects of bioregion on cow growth and size. Genetic trends indicate that the efficiency of growth improved, but was associated with a decline in reproductive characteristics. Indiscriminate selection for growth traits in cattle adversely affects reproduction. The current data indicate that cows of medium size had the best reproduction rates. Acceptable reproduction of larger cows can be achieved with improved management and strategic feed supplementation, although more costly.

Keywords: bioregion, beef cattle, growth, size, reproduction, efficiency

1. Introduction

Sustainable livestock production is imperative on the African continent, to reduce poverty and ensure household food security. It is estimated that edible products from animal origin

account for more than 40% of the total value of South Africa's agricultural output [1]. Only 15% of South Africa is suitable for arable farming, and more than 40% of the remaining 85% receive less than 375 mm rain per annum [2], which explains the relatively low agricultural production potential of the Southern African region. The South African National Strategic Plan for Agriculture endorses the fact that there is very little room for horizontal expansion of agriculture, due to environmental constraints [1], so increased agricultural production can only be achieved by improving the efficiency of production [3] and exploiting vertical integration in regions with a moderate or higher agricultural potential [4, 5].

Long term improvement of the efficiency of animal production can only be achieved through the identification and selection of genetically superior animals for breeding purposes [3, 6]. Selection can be done based on a combination of pedigree information, appearance, and performance recorded information and breeding values [7]. Beef cow efficiency will be highest when cow size is tailored to the environment and the animals are well adapted [8–10]. Cow size has an important influence on the way the cow responds to its production environment [11] and the adaptability of the animal [8]. Adapted animals are tolerant to adverse environmental conditions and are able to maintain reproduction efficiency [6]. In order to improve beef cow efficiency in Southern Africa it is therefore important to optimize cow size, adaptability and employ effective management practices.

The adaptability of beef cattle in extensive production systems is critical and genetic gains in this regard can be best achieved by implementing some beef industry recommendations as listed in [12] namely:

- Identification and characterization of the major beef cattle production environments, and their respective nutritional, physical, climatic, management and economic characteristics,
- Defining the major physical, biotic, social and management stressors in each beef production environment.

2. Cow size and adaptability

The environmental and genetic factors that influence mature cow size include nutrition and management functions, as well as climatic factors such as rainfall and temperature and temporary environmental effects such as differences in fill when weighed and other climatic factors may also influence mature weight [3]. Mature cow weight reflects differences in size associated with skeletal size and lean growth, as well as fatness [13]. The genetic proportion of mature cow weight is mostly due to additive genetic variation, but there are differences in opinion about exactly when cows reach mature weight, e.g., at either 4.5 years, 6.5 years as in Ref. [14], or about 7 years of age [15]. It is difficult to determine exactly when animals stop growing [16], but it is accepted that cows accumulate most of their final weight at 4-years of age and final height at 3-years of age [13].

Several authors have made suggestions about which mature size should be optimal for a particular environment. The significant influence of cow size on production efficiency is also the

reason why traits such as mature weight, height and length, are included in selection criteria [13]. In the late seventies and eighties there was an international trend to select for larger cattle [17], resulting in a net increase in growth rate, but it had a negative impact on female fertility traits [18].

The maintenance overhead is one of the most important factors that determine the biological efficiency of beef cattle, for example an adult cow require more than 50% of her total energy intake for maintenance [11]. Kleiber's theory, however, states that metabolic weight = (live weight)$^{0.75}$ [19]. Larger cows therefore consume more nutrients than smaller cows but the percentage additional nutrient requirement of larger cows are less than its additional weight as a percentage. For example, a cow with mature size of 545 kg weighs 20% more than a 454 kg cow, but its maintenance requirements are only 13% higher [20].

The results of [21] suggest that when nutrient availability is limited, breeds with a moderate genetic potential for growth and milk production are generally more efficient because of higher conception rates. Similar results were reported in [10] in extensively managed Santa Gertrudis cattle in a semi-arid environment. At high levels of nutrient availability breeds with the highest genetic potentials for growth and milk production are most efficient because feed availability is sufficient for the genetic potentials to be expressed. Cow efficiency is thus maximized at a level of feed intake that do not limit reproduction and also provides sufficient energy for milk production to meet the growth potential of the breed as expressed in the calf [21].

3. Functional efficiency and cow size in semi-arid regions

The Bonsmara cattle breed and the concept of "breeding for functional efficiency" was coined by [8], and this concept is effectively employed by the Bonsmara Cattle Breeders Society of South Africa. The Bonsmara is now one of the predominant beef cattle breeds in Southern Africa (>100,000 registered animals; see [27]), and it was created based on a 5/8 Afrikaner and 3/8 Exotic (Shorthorn/Hereford) breeding admixture [8]. Considerable emphasis was placed on the adaptability of the breed. The functional efficiency concept is based on the presumption that selection for phenotypic traits that influence an animal's ability to adapt to the environment, will improve the animal's ability to express its reproductive and productive potential. It was also commonly presumed that specific types or sizes of cattle are better adapted to specific production regions than animals of a different size or type, but this concept was only verified for beef cattle in Southern Africa in a recent study [22].

The natural variation in size of the same species of wild animals occurring in different locations is an indication that nature defines the "right" genetic material for efficiency in different ways in different environments [20]. The influence of production region on cattle production has been investigated by [23], and the notion of an optimal size for a specific environment was previously proposed by several other researchers [8, 11, 17, 20, 24].

The study by [22] which includes records of ca. 12,500 fully registered Bonsmara cows representative of a 20 year period, confirmed that bioregions (Central bushveld, Eastern Kalahari

bushveld, Dry Highveld grassland, and Mesic Highveld grassland) in South Africa significantly influenced the size of beef cows, and also confirmed the existence of an optimal mature cow size in different geographical regions of Southern Africa (**Figure 1**). These findings confirm the importance of the identification of production regions and characterization of optimal body size per region, in order to determine the most suitable areas to purchase breeding animals from, maximize genetic gains and improve production efficiency. A regional livestock classification system was previously published by [25] as illustrated in **Figure 2**, in which areas suitable for different types of livestock were identified.

Biological and environmental features that influence the regional adaptation of livestock as published by [25], remain as valid in modern livestock production, as 60 years ago namely:

- Hereditary differences between the characters determining the productivity of various types of livestock.
- The fundamental physiological phenomena of growth, development, reproduction and production.

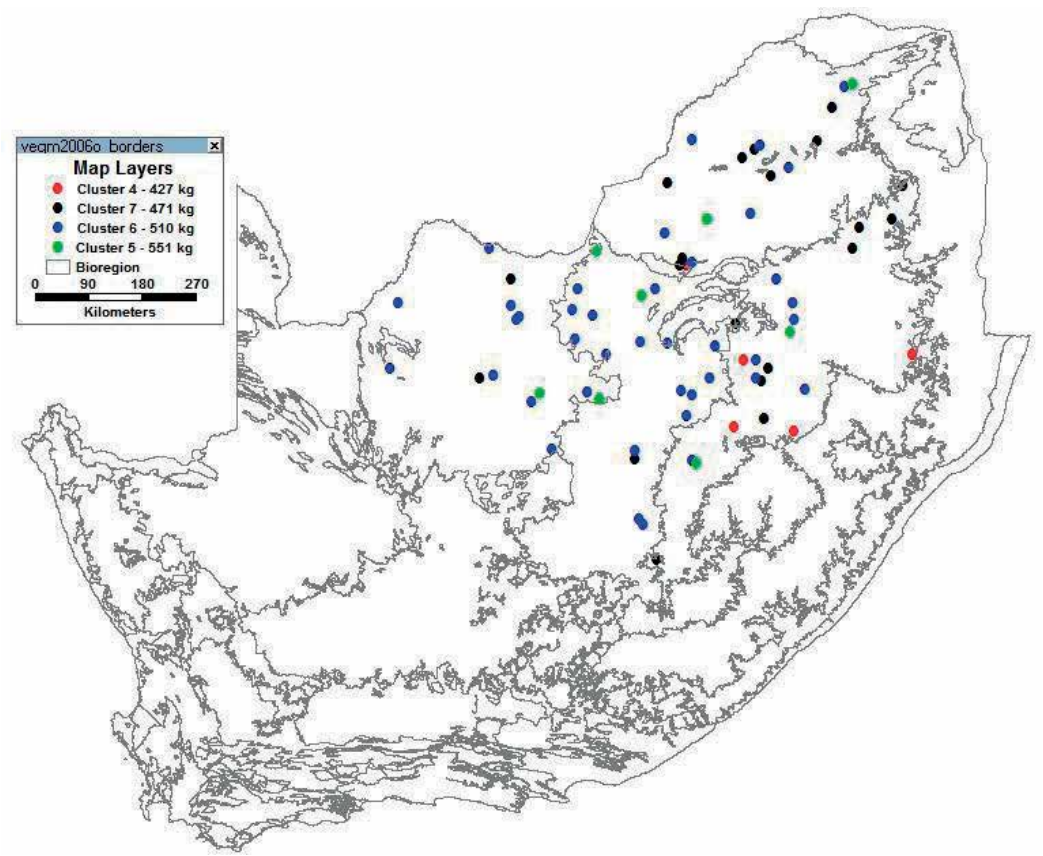

Figure 1. Effect of geographical region on the mean cow size of Bonsmaracows in Southern Africa.

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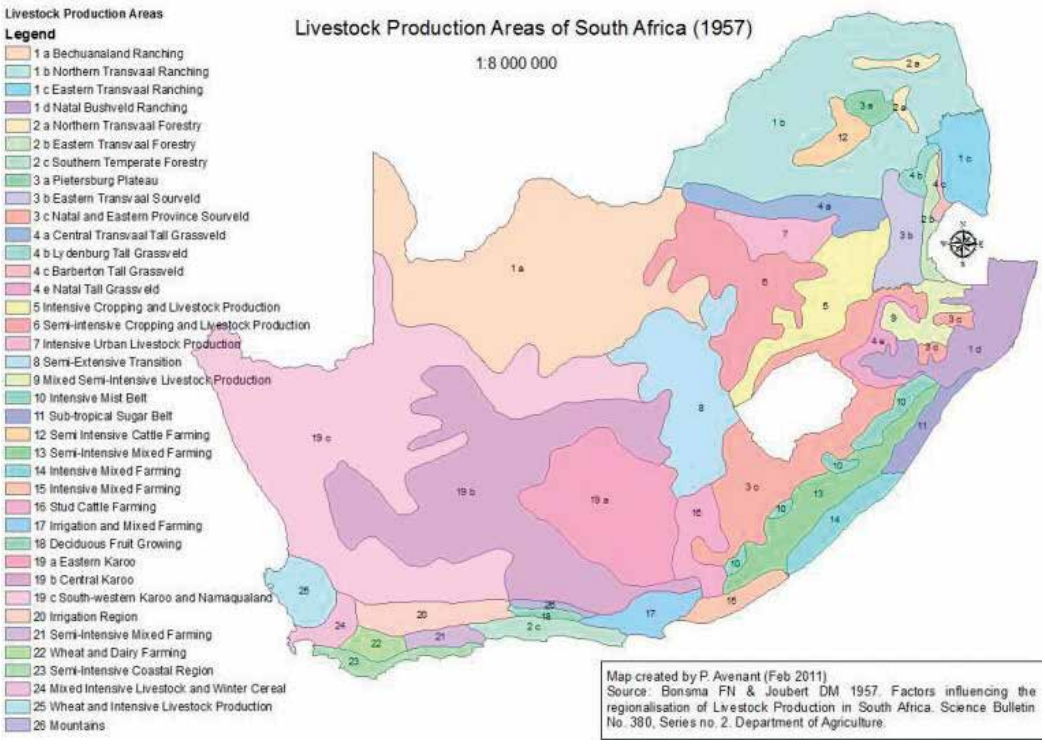

Figure 2. Livestock production areas of South Africa [25].

- The relation between the nutritional requirement of different classes of livestock during successive stages of their existence, as well as their reaction to the climate.
- Geographical and physical features of the various regions and their potential for providing favorable conditions of nutrition in order to promote the optimal expression of the animal's productive ability.
- Information on the distribution of enzootic and epizootic diseases in relation to physical and biological factors which promote the spread of, or assist in its control.

Most pedigree breeds of cattle have a hierarchical breeding structure (**Figure 3**), in which elite breeders furnish breeding material to each other and to middle order breeders. Middle order breeders in turn sell breeding material among themselves and to the lower group of breeders (also referred to as multiplier breeders), but seldom sell animals back to the elite breeders [26]. Analysis of the breed structure of the Bonsmara breed indicates that the combined genetic contribution of elite breeders constitute as much as 30.4% of the genetic composition of this breed. This means that elite breeders have a large influence on the genetic make-up of cattle breeds, which directly affects the types of cattle kept by multiplier and commercial breeders.

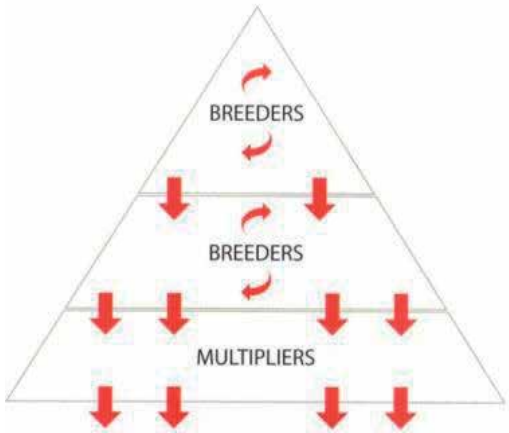

Figure 3. Pedigree breed hierarchy [26].

4. Components of beef cow efficiency

An efficient cow herd is sexually precocious, with a high reproductive rate, low dystocia and has longevity with minimum maintenance requirements [24]. A herd's ability to reproduce in a given nutritional environment is the most important contributing factor to efficiency. Selection goals for efficiency in the cow-calf production systems include early sexual maturity

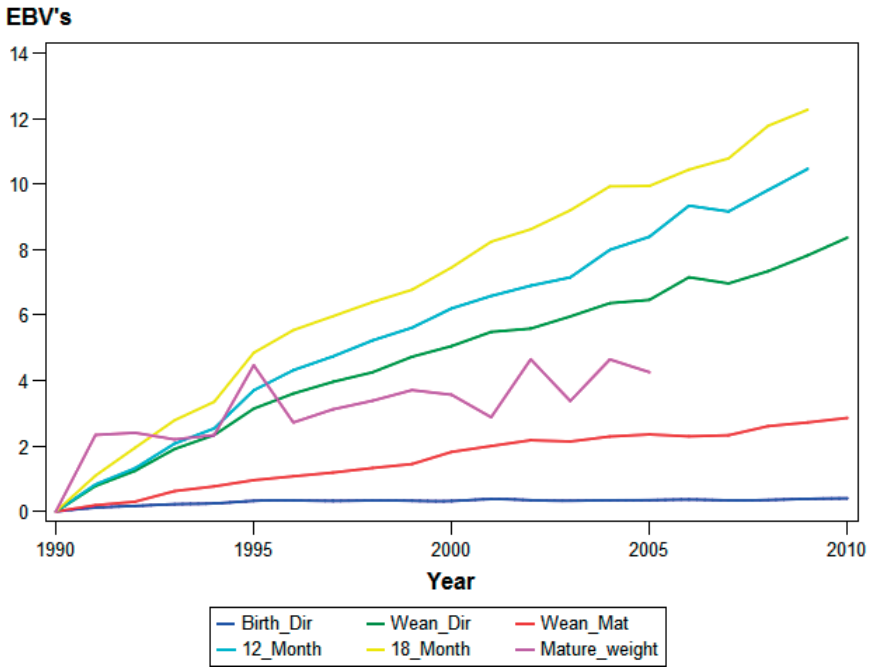

Figure 4. Estimated breeding values (EBV) for growth traits in the Bonsmara cattle breed from 1990 to 2010 (source: ARC-API) (*Birth_Dir ~ EBV for birth weight direct; Wean_Dir ~ EBV for weaning weight direct; Wean_Mat ~ EBV for weaning weight maternal; 12_Month ~ EBV for 12-month weight; 18_Month ~ EBV for 18 month weight; Mature_weight ~ EBV for mature weight*).

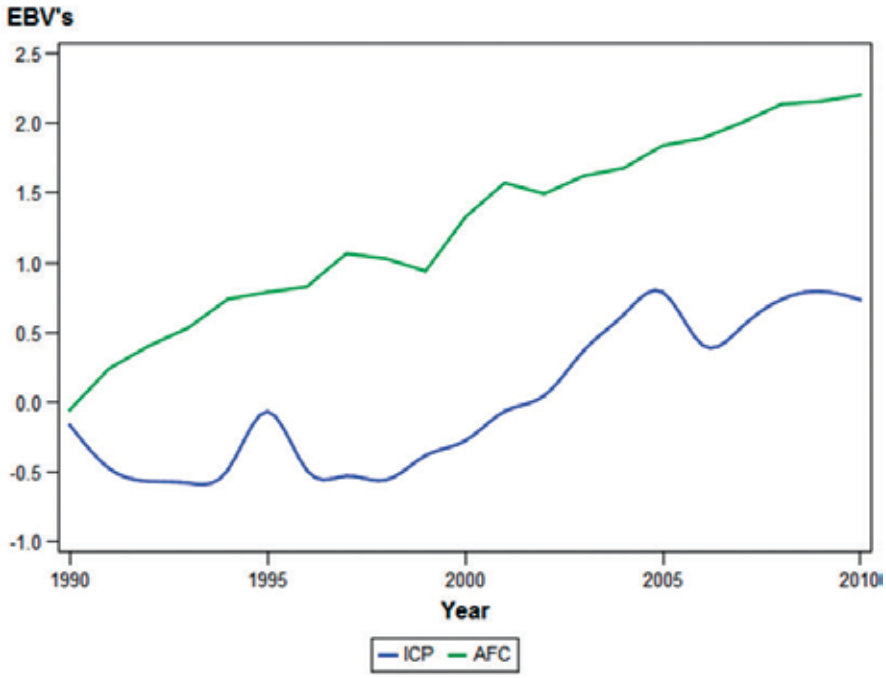

Figure 5. Estimated breeding values (EBV) for reproductive traits in Bonsmara cows from 1990 to 2010 (source: ARC-API) (*ICP ~ EBV of Inter-calving period; AFC ~ EBV for age at first calving*).

with lean growth and minimal increase in mature weight [24]. The most efficient beef cow is therefore the one with the highest milk production that can yearly wean a calf with the growth and carcass characteristics required by the market [20].

Genetic trends for growth and maternal traits in the Bonsmara breed studied by [22] are shown in **Figure 4**. In this study, the genetic trends are presented for a 20 year period (1990–2010), which illustrates the consistent improvements in estimated breeding values for weaning weights (direct and maternal), 12- and 18-month weights, while estimated breeding values for birth and mature weights remained nearly constant. These improvements were obtained in well-managed cattle herds, which generally exhibit regional differentiation in mature size (e.g., optimum body size relative to bioregion, as illustrated in **Figure 1**).

Genetic trends for reproduction traits of the 20 years of Bonsmara breed data studied, are presented in **Figure 5**. Estimated breeding values for both age at first calving and inter-calving period increased since 1990–2010. It follows that the reproductive ability of cows decreased marginally during the same period during which marked progress was made in terms of growth traits, possibly since cow size still exceeded to production potential of the main beef cattle production regions in Southern Africa.

5. Reproduction of extensive beef cattle

Reproduction and calf survival rates are the most important factors that determine the efficiency of a beef herd [10, 24]. In spite of the importance of reproduction it is generally

accepted that in South Africa the calf crop averages between 60 and 65% per annum [27]. Conception rates of cow herds are influenced by a number of interacting factors such as (a) plane of nutrition of bulls and cows, (b) the age of the breeding animals, (c) herd health, (d) libido and (e) semen quality of bulls as well as (f) the ability of cows to conceive and maintain pregnancy [28, 29].

The reproductive ability of a cow is determined by her performance in terms of a number of different reproductive functions that occur throughout her lifecycle. These functions can be divided into component and aggregate traits. A component trait is a single event while aggregate traits are composites of more than one reproductive event [28]. Some of the component traits that can be measured, include time to first oestrus, number of services per conception, pregnancy rate, heifer pregnancy, gestation length, days to calving, age at first calving, calving date, calving ease, calving interval and days open. A combination of these traits are often used to form aggregate traits such as, calving rate, lifetime pregnancy rate, calving success, calf survival and lifetime production. Although these traits might reflect an indication of reproductive performance there are unfortunately no completely satisfactory measure/s of reproduction efficiency [30, 31]. This is due to the influence that the age structure of the herd as well as the prevailing environmental and management conditions have on reproductive recording [28]. Traits that are most frequently used to evaluate reproductive performance are AFC and ICP as well as Reproduction Index (RI), and post-partum anoestrus remains one of the most limiting factors [22, 28, 29].

Age at first calving (AFC) is an important production parameter for commercial beef cattle producers, since it affects the size of cows as well as weight and number of calves produced. AFC also affects the potential annual genetic progress for stud farmers [32]. Beef heifers are generally managed to calve for the first time at either 2 or 3 years of age [32, 33]. Mating heifers earlier may increase dystocia and there are conflicting reports on the lifetime production span of early mated heifers. Some authors reported an increase in the number of calves and weaned kilograms (see [32, 34]), while others reported no increase in the weaned weight, despite the birth of an extra calf [33]. Nevertheless, the success of mating heifers at a younger age depends on nutritional and management levels (see [33]), and most heifers have the potential to reach puberty and breed satisfactorily in such systems [35].

There is a great deal of controversy regarding the use of AFC as a measure of female reproductive ability in the literature. The biggest advantage of AFC is that it can be easily recorded because the birth date of the cow and its first calving date are generally known, while the main disadvantage is that it only represents a single component in the reproductive life of a cow [28]. The general consensus is that in a variable seasonal environment, management decisions often have a greater effect on AFC than genetic merit. Researchers increasingly question the use of AFC, because AFC and the probability of heifers to reconceive are determined by different genes.

It was argued by several that reducing the AFC is one of only a few means of improving lifetime production efficiency in the beef cow herd [33, 34]. Shorter AFC values naturally reduce the generation interval, and thus contribute to the annual genetic gain of the herd [36]. Another common but erroneous belief is that scrotal circumference in yearling bulls may be an indicator of reproductive fitness in female offspring [35, 37]. Scrotal circumference was

therefore often included in selection programs to improve heifer fertility. However, recent datasets indicate that the association between scrotal circumference and heifer fertility traits is low [36, 38].

5.1. Inter-calving period

ICP or calving interval is an aggregate reproductive trait, composed of more than one reproduction event, and is defined as the time that elapsed between two successful calving's [28]. ICP is regarded as an important fertility trait, especially if one considers the importance of reproduction in a calf production system [39]. The ideal would be that every cow should calf every year and that the ICP of a beef cattle herd should be less than 365 days [38, 40]. This means that a cow should conceive within at least 80–90 days after calving, but it is accepted that the ICP in many breeding herds often exceeds 365 days in the tropical or subtropical areas due to high humidity and temperature and lower forage quality [41]. According to the SANBRIS, the current ICP average for the different breeds in Southern Africa ranges between 398 and 477 days. The Hereford and Shorthorn breeds have the shortest (398 days) and the Huguenot the longest (477 days) ICP, while the average ICP of the Bonsmara breed is 405 days [27].

The use of ICP as a measure of reproductive efficiency in a fixed breeding season has been questioned by several authors [28, 30, 42]. The major criticism against ICP as a selection criterion for reproductive performance is the negative correlation that exists between ICP and previous calving date as well as the large influence that the previous calving date has on the ICP [42]. This means that cows that calve early in the season have the longest ICP while those that calve late in the season have the shortest ICP.

The low heritability of ICP is also another question raised. The estimated heritability ranges between 0.02 (see [43]) and 0.12 (see [31]), with a low repeatability of 0.14 [43]. The repeatability estimate for ICP suggests that female culling based on first calving interval is not accurate and there is a risk of culling animals with other desired traits. Selection for shorter ICP's could result in indirect selection for a later age of puberty as cows with the shortest calving interval, are often those who calved late in the season [30]. It also does not take information from the first parity or the end of a cow's life span into account when the ICP of the herd is determined [28].

The analysis of ICP is also problematic because it is only available for cows that calve repeatedly and should therefore be treated as a censored trait. Fortunately ICP is based on the period between two calving's; it can therefore be easily computed with a minimum of data, and this data will be lost from the reproductive information for the first parity as well at the end of a cow's lifespan if no calf is born [28, 29].

5.2. Post-partum anoestrus

Post-partum anoestrus (PPA) is the period after parturition during which cows do not show behavioral signs of oestrus, which is one of the main causes of extended ICP [40]. Although PPA is caused by static ovaries, there might be follicular development, but none of the ovarian follicles become mature enough to ovulate. PPA may be caused by a number of factors, such as pre-partum feeding level as reflected by body condition at calving, post-partum nutritional

status and parity of the cow, suckling interval (see [40, 44]), cow-calving season due to nutritional factors and or light and temperature and dystocia (see [44, 45]), the presence of a bull (see [45]) breed and age of parity also has an influence (see [45, 46]) as well as sire breed.

Although many factors affect postpartum anoestrus, nutrition and suckling are the major influences on the resumption of postpartum ovarian cycles, as it affect hypothalamic, pituitary and ovarian activity and therefore inhibit follicular development [47, 48]. Under-nutrition contributes to prolonged postpartum anoestrus, particularly among cows dependent upon forage to meet their food requirements [40]. The nutritional status or balance of an animal is evaluated by means of the Body Condition Score (BCS) parameter. BCS reflects the body energy reserves available for metabolism, growth, lactation and activity. There is a relationship between energy balance and time to the resumption of postpartum ovarian activity.

Inadequate nutrition cause excessive weight loss, followed by a decrease in BCS and finally cessation of the oestrus cycle. Suckling probably interferes with the hypothalamic release of GnRH and suppresses the pulsatile release of LH which leads to an extended postpartum anoestrus [40]. However, the exact interaction by which suckling extends post-partum anoestrus is uncertain [49]. The huge benefits of 12- or 48 hour calf removal prior to the onset of breeding were clearly demonstrated [47, 48]. This research disclosed significant improvements in conception rates from 55 to 76% in Brahman-type cows in a semi-arid environment. Such strategies are beneficial in terms of beef cattle production, but are not widely employed in Southern Africa.

Other factors that influence the anoestrus period after calving and cause a longer inter-calving period are: general infertility, uterine involution, short oestrus cycles and post-partum anoestrus [45]. Management practices play an important role in the ICP of a herd and the following practices may decrease PPA:

- Introduce a short breeding season.
- Make use of BCS to monitor nutritional management.
- Minimize dystocia distress.
- Use a sterile teaser bull with cows during the early postpartum period before the breeding season starts.
- Synchronize oestrus.
- Decrease suckling stimulus.

Although there are numerous objections to the use of inter calving period (ICP) as a measure of female reproductive performance, there is no alternative to ICP as a measure of reproductive performance [50].

6. Maternal component of growth

Growth traits like birth and weaning weights are determined by the animal's own additive genetic merit as well as the maternal component, which can be further separated in an

additive genetic and a permanent environmental component [51]. The maternal component mainly represents the dam's milk production and mothering ability, although the uterine environment and extra-chromosomal inheritance may also have an effect. The dam's genotype therefore has an effect on the phenotype of the young through a sample of half her direct, additive genes for growth as well as through her genotype for maternal effects on growth [52].

Postnatal calf growth and physiological development are initially influenced by stimuli experienced *in utero* [53]. Maternal nutrition therefore potentially affects not only cow productivity but also post-weaning calf productivity [54]. Protein supplementation during late gestation, as well as increased total nutrient supply throughout gestation, may increase calf birth weight [54]. Another major component of the maternal environment created by the dam is the nutrition the calf receives through milk. There is a positive relationship between the breeding value for milk for the dam, actual milk production and the weaning weight of calves [55]. A high correlation (0.8) was reported between direct milk yield and maternal weaning gain (see [56]).

Milk intake also influences forage intake of nursing calves, e.g., calves of dams with lower milk production are more reliant earlier in lactation, on alternative feed sources of lower nutritional value than milk [57]. Calf body weight and forage dry matter intake are correlated with calf milk intake, and nursing calves generally become increasingly dependent on forage after 60–90 days of age to maintain normal growth. It follows that the forage quality of rangeland systems affect growth rates of calves through influences on the milk yield of dams and quality of the forage portion of a calf's diet.

7. Effectiveness of selection for reproduction efficiency

Fertility is a complex trait that has many components [28, 29]. Both male and female traits contribute and show considerable variability. Selection for both male and female fertility is therefore desirable [56]. Although the aim is usually to maximize the reproductive potential of beef cattle, more is achieved by optimizing rather than maximizing reproduction because the gross margin per cow increases parallel with the calving rate, but the margin per cow does not necessarily show the same response [58]. Fertility traits are heritable, but relatively few heritability estimates have been reported for fertility in beef cattle [28, 38]. In a review of fertility traits the heritability estimates for fertility ranged from ≤0.10 to ≥0.60 [38]. Unfortunately genetic improvement of fertility is hampered by a lack of information, low heritability and the delayed expression of the trait.

The heritability's of fertility traits are difficult to estimate because the expression of the reproductive potential is often constrained by management systems [29, 56]. Moreover, the underlying genetic merit for fertility is often not expressed, due to the threshold nature of fertility traits. There are only two outcomes possible for successful reproduction: Whether the cow is pregnant or not, degrees of pregnancy are not observable. The environment has a strong influence on which side of the threshold trait an individual falls [35]. The general consideration is that selection has a limited potential to improve fertility in beef cattle, while improvements in cow

and environmental management hold much promise to optimize cow reproduction [22]. One of two approaches is often recommended when selecting for improved fertility [35] namely:

Step 1. The *direct* approach involves the physical selection for fertility traits. This should include traits such as scrotal circumference, age at puberty, age at first calving as well as calving date and the proportion of heifers in production at a given age. The use of any prospective fertility trait depends on the ease of measurement and the inherent relationship with fertility.

Step 2. The second or *indirect* method proposed is to use an array of traits that indirectly affect fertility, such as milk production, growth rate, calving ease and body condition. Selection for optimum combinations of these traits should create a favorable "genetic environment" for fertility.

8. Influence of selection for growth on beef cow efficiency

Growth traits are highly heritable, with heritability's ranging from 0.24 to 0.61, so fast genetic progress is possible when animals are selected for growth rate [59]. Selection for growth is complex, since traits like birth and weaning weight are determined by the animal's own additive genetic merit as well as the maternal component, which can be further separated into an additive genetic and a permanent environmental component [51]. It is well known that selection for a higher growth rate eventually increases the mature size of animals, which is due to the positive correlation between weights at different ages [59]. There is also a negative correlation between mature size and age of maturation, which means that selection for larger size in the long run increases the time taken to reach maturity.

Genetic change in the shape of the growth curve is limited by the degree of genetic flexibility in the shape of the curve, which depends on the degree of interdependence of the size, rate and inflection of the growth parameters [3, 60]. Although theoretically possible, the basic shape of the sigmoidal growth curve as well as the sequence of physiological events remains virtually unchanged. The rate of these processes has however increased remarkably over the past few decades [3]. In fact, selection for increased body weight or growth rate may have an adverse effect on body composition, fertility and survival rate [27]. It was suggested that selection should rather be focused on increased feed efficiency because it may lead to fewer adverse effects. Some researchers also postulated that selection for growth and efficiency may have reached the physiological limits of animals to cope with the demands of maintenance, accelerated growth, development, adaptation and reproduction [3].

9. Growth rate and reproduction

Information on the effects of selection for body weight or growth rate on reproductive fitness in cattle is unfortunately limited [27]. In a fundamental theorem of natural selection in the 1930s it was already postulated that reproductive fitness and body weight will be near the peak of fitness in a natural population [61]. However, when selection for growth takes place, the population is no longer in a natural equilibrium, so the reproductive fitness may in fact

decline when the mean of a population is moved in either direction due to selection pressure [62]. The antagonistic relationship between fertility and milk production in dairy cows and the resource allocation theory support this theory [63]. The general consensus is that selection for increased body weight or growth rate may have an adverse effect on fertility [27] for the following reasons namely:

- Increased infertility is the result of the deviation from an optimum body weight that is associated with an optimum degree of fitness.
- Pleiotropic genes with opposite effects on growth rate and fertility may become important as a result of prolonged selection.
- Major changes in body weight or growth rate may upset the natural homeostasis and endocrine balance which developed in each species over its evolutionary history.
- Selection for increased growth rate may result in indirectly selecting for feed intake and this may lead to the breeding of animals with a predisposition for high feed intake. Gluttonous animals can become obese at maturity, which may influence fertility.

There is therefore a concern that selection for high growth rate might have negative effects on the fertility of cows [64]. However, contrasting results have been published which indicate that cows with a high pre-weaning growth, reared more calves over their lifetime, had lower calf mortalities and also calved earlier than cows with lower pre-weaning growth [65]. In another unrelated study the reproductive performance of Angus females selected for a high growth rate was similar to those of females where there was no deliberate selection pressure at all. The EBV trends obtained and presented in **Figures 4** and **5** for growth and reproduction traits in Bonsmara cows, indicate a negative correlation and warns against excessive selection for growth traits in extensive beef cattle, especially if the natural resources are limited [22].

10. Conclusions

This study investigated the effects of different bioregions in the semi-arid parts of Southern Africa on the growth, size and reproduction efficiency Bonsmara cows. This study employed novel techniques to investigate the influence of production environment on the growth, size and reproduction efficiency Bonsmara cows. Results indicate that bioregions affect the growth, size and reproduction efficiency of beef cows, and provide evidence for the existence of an optimal cow size for different bioregions. Results revealed a complicated relationship between bioregion and the growth, size and reproduction efficiency of Bonsmara cows. The proportion of variation in cow growth traits due to the regional distribution of cows, depended on the physiological stages of growth, e.g., birth, weaning, 12- or 18-month growth stages. In terms of production efficiency, weaning- and yearling weights as well as AFC and RI were influenced most by differences in regional distribution of cattle. Management practices and breeding objectives have a major effect on the efficiency of beef cow production

efficiency. The effective implementation of management practices, such as the provision of nutritional supplementation at weaning and yearling age, limits the negative influence of regional effects on cow growth and size.

Genetic trends indicate that the efficiency of growth improved remarkably during the past 20 years in the Bonsmara cattle breed. However, improvements in growth and efficiency, were associated with a decline in reproductive characteristics. Reproduction efficiency is the single most important aspect of beef cow efficiency and breeders should guard against indiscriminate selection for growth traits, which may adversely affect reproduction performance, especially since much research endorse the existence of a negative relationship between growth and reproduction traits. Although the common belief is that smaller cows reproduce better in more resource constrained regions, the current data indicate that composite type cows of medium size had the best reproduction rates. The reproductive ability of larger size cows improves markedly with improved management and strategic feed supplementation.

Acknowledgements

The financial support from the Red Meat Research Development Trust of South Africa is acknowledged. The researchers are grateful for the technical support of the staff at the Agricultural Research Council at Irene, South Africa.

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Chapter 4

Twin Calving and Its Connection to Other Economically Important Traits in Dairy Cattle

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.72905

Abstract

The occurrence of twin calving in Holstein Friesian cattle is 3–5%. Reproductive problems found before and after the time of calving lead to serious economic losses. The authors' aim was to form a compilation of data regarding the cause and effects of twin calving on dairy cows by comparing the reproductive performance of cows before and after calving twins vs. single calves and also analyzing the milk production of dairy cows both before and after twin calving. Cows that would have twins became pregnant earlier, probably because of their better health and fertility, with shorter gestations and calving interval. However, after calving, cows that had twins showed poorer reproductive performance. The results show that twin-calving cows had better condition prior to calving, resulting in an earlier successful twin calving. However, the economic losses during parturition, metabolic disorders of the cow, and low vitality of the twin calves, coupled with the decreased fertility and elevated culling rate in cows after twinning, may discourage breeding twins in dairy cattle.

Keywords: Holstein Friesian, twinning, reproductive traits, milk yield, fertility, culling reason

1. Introduction

Investigations into the topic of bovine twin calving were first published in the early 1900s. Both cattle breeders and experts were interested in how cows that are normally uniparous animals can give birth to two or more healthy calves. In the 1930s [1], an important goal of research into twin calving was highlighted: raising identical calves in different experimental conditions [2]. Twinning has remained a topic of interest for researchers as it carries with it many pros and cons for dairy and beef farmers. Approximately 10% of all pregnancies in dairy cattle result in twins. However, in beef cattle, the incidence of twins is less frequent [3, 4].

Superficially from an economical viewpoint, the idea of an excess number of calves per calving can seem advantageous. Nevertheless, when the outcomes are analyzed, twin calving has several negative consequences. Increased rates of abortion [5], dystocia [6], higher mortality of the calves around or during parturition [7], and increased rates of placenta retention [8] are seen far more frequently in twin-calving cows. Twins born tend to be less developed than single-born herd mates of the same age, they have higher mortality rate [9], and rebreeding the dam can be delayed [10], leading to a longer calving interval and decreased profits. Use of sexed semen has been shown to result in a lower frequency of twin pregnancies [11]. However, this lower twinning rate is due to damages caused to spermatozoa by the physical forces during the sexing process, which in itself has other negative effects that are well known such as decreased conception rates [12] and is not dependent on the dam.

Another important aspect to consider is the qualities of the dams with a predisposition to twin calving, as the cows with higher lactation performance and better fertility tend to be more predisposed to multiparous pregnancies [6]. And hence some of the most advantageous and desirable traits in a dairy cow can lead to twinning, which is overall considered to be a negative trait.

2. Factors impacting twinning

It has been well documented that [13–15] the natural frequency of twin calvings is influenced by a wide variety of genetic and environmental factors ranging from 0.5 to 4% depending on these factors.

Mainly, environmental and genetic factors influence the number of twin Holstein Friesian calves born every year. The factors having most influence over multiple births are age, parity, seasons and climate, feeding, milk yield, hormonal influence, and genetic predisposition.

2.1. Genetics

It has been shown that there is a far higher incidence of twins in dairy cattle as opposed to beef cattle [16]. The frequency of twin births also shows variation by breed [17], with incidences of multiple ovulations much more frequently observed in Holstein Friesian cows than in other dairy cattle breeds [15].

The results drawn from a model calculation estimating the genetically determined number of calves per calving (or litter size), the real incidence of twinning, and the percentile contribution of triplet and quadruplet pregnancies are shown in **Table 1**.

2.2. Age

Age is also a contributing factor when dealing with the rate of occurrence of twin and multiple births. Cows that have had several lactations have a higher number of twin and multiple births compared to that of heifers [19].

Multiples, %	Mean litter size (realized number of calves)	Triplet, %	Quadruplet, %
5	1.050	0.0	0.0
10	1.101	0.1	0.0
20	1.203	0.3	0.0
30	1.309	0.9	0.0
40	1.421	2.1	0.0
50	1.546	4.6	0.0
60	1.696	9.4	0.1
70	1.894	18.3	0.5
75	2.020	24.6	1.2

Table 1. Average litter size and percentage of triplet and quadruplet births predicted from percentage of multiple birth [18].

Twinning not only increased from first to second parity, but also from second to third parity, to then more or less plateau for the remainder of the reproductive life of the cow [9].

Older cows have a tendency to carry twins more frequently due to the cows' maturity and the increased occurrence of polyovulation [20]. The increase in incidence of double ovulations is approximately linear with increasing calving numbers [21]. One explanation that has been proposed for this is that in twin pregnancies the increase in embryonic mortality in younger cows is due to the uterus having insufficient functional capacity. The total area of the uterus in heifers and in young cows is usually not sufficient to support several embryos [22].

2.3. Seasonality

Generally, it is found that more twin and multiple births take place in summer, spring, and autumn than in the winter months. The percentage of pregnancies that were twins was found to be 2.41% in summer, 2.04% in spring, and falling to 1.79% in autumn [17, 23, 24]. A study in the USA (Minnesota) [9] showed that the percentage of twin births in April–June was 8.3%, July–September was 9.7%, October–December was 5.2%, and January–March was 2.3% in the northern hemisphere.

The postpartum conception rate was higher in autumn than in spring due to the higher feed quality in the autumn months and the lower temperature (so that heat stress is not a problem). The opposite was found to be true [25] when dealing with beef herds, in which twin births were seen to be 50% higher in autumn than in spring [19].

The University of Wisconsin performed an investigation on the North American sires and found that the highest rate in twinning was in the lead up to the summer solstice and the lowest was at the winter solstice [26]. One cause of most frequent summer twinning ($p = 0.002$) could be that during the autumn months, the weather is cooler, and the hot summer months that would subject the cow to heat stress, which could damage the embryo, are over [27]. The survival rate of the embryo or the fetus and the ovulation rates determine if there will be a single calf, twin calves, or multiple calves born [28]. During the study

period, 1281 (3.94%) twin births occurred. The ratio of twin deliveries on the farms varied between 3.43 and 4.35%.

2.4. Nutritional influences

We see that food intake is reduced with the rise in temperature in the summer, along with the decline in reproductive performance and milk yields [29]. Better feed quality supplied in the autumn and optimum temperature (absence of heat stress) contribute to multiple ovulations in cows [30].

Cows with high milk yield show a threefold higher frequency of double ovulation than cows with medium to low lactation performance. Flushing could potentially be given as the reason for this [21].

Quality of feeding has a huge knock-on effect throughout the life of a cow: before fertilization, prepartum, and postpartum. Nutrition plays a great part with the aim of getting the maximum energy intake from the silage and feedstuff to optimize milk production and fertility performance of the herd. This goes hand in hand with good management and good farming practice.

Good nutrition can also lower the risk of postpartum disorders like ketosis, mastitis, and milk fever, which were associated with high reduction in milk production [16].

2.5. Milk yield

Milk production is the primary factor affecting the incidence of double ovulation in lactating dairy cows [21].

High milk production near the time of ovulation can increase the incidence of double ovulation, which would therefore subsequently result in an increase in twinning. Current dairy management strategies are aimed at maximizing the milk production of the cow. This intensive feeding strategy would in turn increase the incidence of double ovulation in the dairy cattle population in general. It is therefore not unexpected that given the annual increases in milk production that there should be an overall increase in twinning rates as well.

2.6. Hormonal influences

It was found [31] that the rate of twinning increased with increases in milk production, incidence of cystic ovarian disease, and the use of common pharmaceuticals, including gonadotropin-releasing hormone (GnRH), prostaglandin (PGF2α), and antibiotics. For farmers and the milk industry, only a functional cow with regular and healthy offspring is profitable. Any kind of disorder in relation to the reproductive system will cause losses, by increasing the number of required inseminations and elongating the calving interval. To avoid or at least to reduce these issues, a hormonal treatment could help to reach a successful pregnancy [32, 33]. For the "OV-Synch method," the cows get two intramuscular injections of gonadotropin-releasing hormone (GnRH) as well as one prostaglandin (PGF2α) injection in a specific order to synchronize the ovulation of a herd [21]. Another benefit beside the synchronization is the use of this injection combination to treat cysts of the ovaries [34]. Due to the effect of hormones

on the cows' body, 14.1% of the cows have shown a double ovulation, and with that, in 5.2% of the cases, a twin pregnancy was detected [21].

3. Reproductive traits in twin- and single-calving cows before calving

Data from 393,002 parturitions were recorded along with the cows' performance in the following production cycle [30]. Data were collected from 145,457 Polish Holstein Friesian cows. The animals initially calved between 2000 and 2012 and were either in use or culled by the end of 2013. Among the factors analyzed, the cow's age had the strongest effect on the rate of occurrence of multiple pregnancies, showing multiple pregnancies to be much more frequent in cows than in heifers. Each consecutive lactation raises the chance of multiple ovulations, and occurrence of twin births increases linearly with the frequency of double ovulation [21, 35]. The incidence of twin pregnancies goes from 1% in maiden heifers to about 10% in following lactations. The rate of twin pregnancies increases the most between the first and second lactation. After the second lactation, the incidence increases to a lesser extent [23]. It should also be noted that heifers have an increased mortality of one of the fetuses in multiple pregnancies, due to the high nutrient requirement of the growing body of the heifer coupled with the demands of the twin fetuses [36].

In an analysis [37] of 4000 cows calving between 2000 and 2010 in a herd in northern Hungary (**Table 2**), ages were compared at first breeding, at first conception, and at first calving of twinning and nontwinning cattle. The average age at first breeding at the farm was 17.9 months (544 days), and no significant difference was found between future twinning and nontwinning heifers. The average age of first conception was 18.3 months (557 days). There was no significant difference between nontwinning and twinning heifers. On average, heifers became

Reproductive traits	Cows with single calving/n		Cows with twin calving/n		p-Value
Age at first breeding, month	17.88	3569	17.92	392	0.753
Age at first conception, month	18.32	3410	18.34	376	0.868
Age at first calving, month	27.45	3632	27.47	395	0.830
Calving to first service interval BF, day	73.90	6208	73.92	722	1.000
Calving to first service interval AF, day	73.59	5207	78.09	275	0.043
Open days BF	109.3	5318	104.6	642	0.047
Open days AF	109.3	5207	123.1	275	0.001
Gestation period BF, day	278.7	9940	274.0	724	<0.001
Gestation period AF, day	278.8	5207	279.0	275	0.511
Calving interval BF, day	404.2	5698	392.2	680	<0.001
Calving interval AF, day	397.5	5207	410.3	275	<0.001
Total life span, month	60.12	3581	75.97	386	<0.001

Table 2. Reproductive traits of cows with single and twin birth before (BF) and after calving (AF) [37, 38].

pregnant by the 13th day of service if the first or second insemination was successful. On analysis of age of first calving, it was demonstrated that on average the age of first calving in pregnant heifers was 27.5 months, and there was also no significant difference between twinning and nontwinning heifers. The lack of deviation is due to the management system and when the farmer chose to inseminate them.

The calving to service period prior to a viable pregnancy was on average 73.9 days, showing no significant difference between twinning and nontwinning cows. The cows that were nontwinning took 73.6 days.

There is a statistically proven difference between twinning and nontwinning cows ($p = 0.047$) in the length of open period, which was 109.3 days for nontwinning and 104.6 for twin-calving cows, meaning that twin-calving cows required a shorter service period by 4–5 days than nontwinning cows, due to their better fertility and higher chance for conception.

Cows carrying twins had an average gestation length of 274.0 days, which was confirmed to be shorter than their herd mates carrying single calves for 278.7 days. There was a significant ($p < 0.001$) difference in this study of nearly 5 days due to the type of calving. The average length of gestation was 276.3 days. The significant contributing factors to the total variance were the following: type of calving, calf gender, and calving season, 64.22, 17.32, and 10.92%, respectively.

Calving interval of the herd was 398.2 days. A relevant difference was detected between the twin-calving and single-calving cows. The calving interval in twin-calving cows was 392.2 days, shorter than the calving interval in nontwinning cows, which was 404.2 days. A difference of 12 days is significant ($p < 0.001$).

This farm study assumed that cows that would carry twins have a better body condition, which leads to better fertility provided the housing and feeding all the cattle receive are the same. It was concluded that the beginning of the breeding phase for young animals is dependent on the decision of the farmer and it will be similar for every heifer on that farm. The first part of the investigation was concerned with events only occurring once in life of a heifer. However, later characteristics that occur repeatedly in life of a dairy cow were analyzed, and in all cases, the characteristics were taken into consideration before calving, demonstrating differences between the length of the calving interval, open days, and gestation during the herd's life. This shorter calving interval can be seen as advantageous in a dairy herd.

4. Production traits in twin- and single-calving cows before calving

In another study [30], the rate of occurrence of multiple pregnancies was found to increase noticeably ($p \leq 0.01$) as the milk production level of the cows increased. Higher yielding cows are predisposed to double ovulations, which directly affects the incidence of twinning [39]. High energy diets offered in the early lactation are a contributing factor in the rate of double ovulations [23, 24]. For breeders, it is worth noting that the rate of twin births increased significantly ($p \leq 0.01$) from 0.43% in heifers between 2000 and 2003 to 0.77% in heifers

between 2010 and 2012. This is thought to be linked to increased production levels of the active population, because the yield of cows in their first lactation in 2000 was 5969 kg compared to 8215 kg for cows in their first lactation that calved in 2012.

Milk yield is positively correlated to frequency of multiple pregnancies [3, 4]. In an analysis of 91,008 calvings between 1974 and 1985, it was shown that twinning was much more frequent in Holstein Friesians compared to the Polish Black-and-White breed.

5. Reproductive traits in twin- and single-calving cows after calving

In **Table 2**, reproductive characteristics after calving are also presented with respect to the time from the calving to the service period [38]. The average number of days in the resting period on the farm was 75.8. The number of the calves had a strong effect on the length of the studied period ($p = 0.043$). The cows that had single calves took 73.6 days compared to the cows that had calved twins 78.0 days to recover.

The service period was analyzed in a separate study of the same herd data [40] and the average service period length was 44.6 for the herd overall (**Figure 1**). The single-calving cows had a shorter (44.4 days) service period than twin-calving cows (51.2 days), but this difference was not considered statistically significant ($p = 0.111$). It was found that roughly a third of the cows were put in calf by the very first insemination. Fifty percent of the cows that had single and twin calves were put in calf by the 40th and 48th day of service period, respectively. However, it was found that this 8-day difference was not a statistically proven difference ($p = 0.653$)

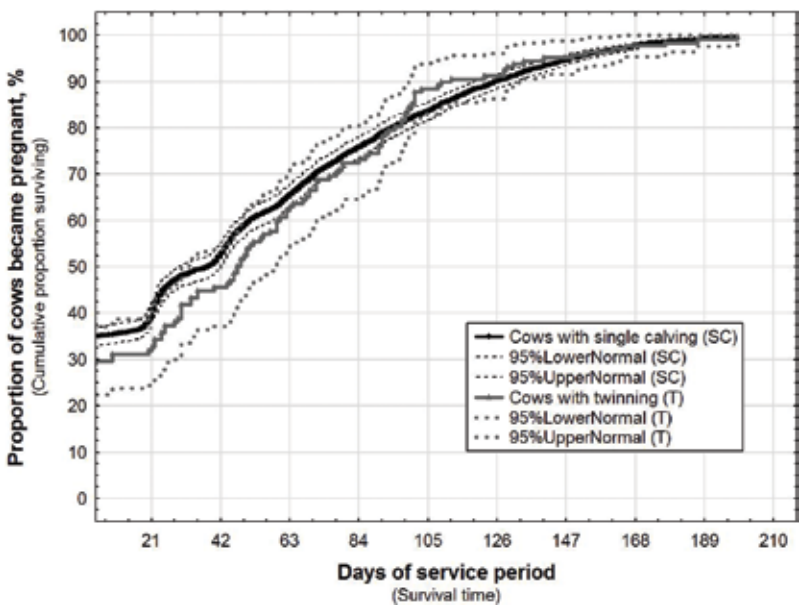

Figure 1. Cumulative proportion of cows that became pregnant on the course of insemination period [40].

between the groups of cows. This shows us that cows with single calvings and twin calvings respond in a similar way to rebreeding.

On examination of the number of open days after calving [38], a relationship was found to twinning (**Table 2**). The average number of open days was 116.1. Based on the number of the calves, it was significantly different ($p < 0.001$). For the single-calving cows, there were 109.3 open days on average, whereas for the twin-calving cows, it took 123.1 days. In case of the twin-calving cows, the open days were 7 days more than the farm average.

Analyzing gestation length after calving [38], it was found that the average was 278.9 days. There was no significant difference between twin-calving and single-calving cows.

The length of the calving interval after calving was on average 403.9 days. However, there was significant deviation from the average in the twin- and non–twin-calving cows ($p < 0.001$). The calving interval following a twin calving was 6 days longer than the calving interval of single-calving cows, and 13 days longer than the farm average. This is thought to be because after the twin calving the involution of the uterus takes longer, increasing the time between two calvings [6].

Another study [14] analyzed the reasons for culling in a herd of 23,588 cows of a local breed, 17 of which were improved with Holstein Friesian between the years 2000 and 2008. After having single calves vs. having twins or triplets, the culling patterns reveal some of the reproductive issues associated with multiple pregnancies. After single pregnancies, udder diseases account for 11.83% of culling, and fertility and reproductive diseases account for 34.58% of culling, whereas these figures are higher in multiple pregnancies with udder diseases accounting for 13.54% of culling and fertility and reproductive diseases accounting for 37.99% of culling (**Table 3**).

Reasons for culling	**Proportion of production cycles interrupted due to selling or curling depending on type of pregnancy**	
	Single pregnancy (%)	**Multiple pregnancy (%)**
Production cycles interrupted due to selling or culling (n, %)	20,548 (27.74%)	458 (40.71%)
Sold for further breeding	13.81	9.61
Low yield	3.82	2.62
Udder diseases	11.83	13.54
Fertility and reproductive diseases	34.58	37.99
Infectious diseases (leukemia)	2.79	1.97
Old age	1.09	1.31
Metabolic and digestive diseases	2.06	3.93
Respiratory diseases	0.12	0.00
Diseases of the locomotor system	3.22	4.37
Accidents	21.27	17.25
Other	5.14	7.42

Table 3. Culling patterns in cows after single and multiple pregnancies ($\chi^2 = 28.26$) [14].

This study also showed that triplet-calving cows have even worse reproductive issues than twins, showing that the calving interval was 416 days in single-calving cows, 430 days in twin-calving cows, and an excessive 487 days in cows that had triplets. The rest period after calving was shown to be 87 days for single-calving cows, 96 days for twin-calving cows, and 104 days for cows that had triplets. Similar delays were found in the service period, which was found to be 45 days in single-calving cows, 52 days in twin-calving cows, and 93 days in cows that had triplets. The percentage of successful first inseminations was found to be 49.11 for single-calving cows, 45.76 for twin-calving cows, and 28.57 for cows that had triplets.

The effect of twin calving on open days in Holsteins was studied in the USA [41]. The effects of twin calving in Holsteins were studied from a compilation of calving records from the Eastern Artificial Insemination Cooperative. Cows were grouped according to whether the twin calving was associated with dystocia. Records of each cow that had a twin calving were paired with records of a single-calving control herdmate. In the twin group of 175 cows associated with dystocia, after twin pregnancy, open days following twin calving were increased. In the twinning group of 367 cows with no dystocia at twinning, open days after twinning were increased by roughly 22 days, indicating a negative economic effect of twin calving suggesting increased rates of twinning via artificial selection or artificial induction of twinning in dairy cattle would not be desirable.

6. Production traits in twin- and single-calving cows after calving

A study of the same dairy farm in northern Hungary [40] compared the total lactation performance data of 10,666 cows, in detail, examining the most economically important traits such as milk yield (kg), fat yield (kg), and protein yield (kg) and taking into account factors such as whether the cow had a single or twin calving, the proportion of Holstein Friesian genetics, the season the calving occurred in, the number of lactations, and the year of calving. The average total milk yield produced was 7140 kg, a noticeable difference was seen in comparison of single-calving and twin-calving cows in favor of single-calving cows ($p = 0.013$). On average, single-calving cows were found to produce 7390 kg of milk, whereas the twin-calving cows were found to produce 6890 kg. In terms of the type of calving, it was shown that cows that had twins yield on average 500 kg less milk than single-calving cows. As mentioned above, not only was total lactation yield (kg) evaluated but also fat yield (kg) and protein yield (kg) were evaluated. Regarding the fat yield, a meaningful distinction was recognized between single-calving cows and twin-calving cows ($p = 0.030$); single-calving cows gave 17 kg more fat.

The overall standard lactation milk yield is 6685 kg. When the milk yield ($p = 0.002$), the fat yield ($p = 0.001$), and the protein yield ($p = 0.004$) were examined, they were found to have significant differences based on the type of calving. Twin-calving cows produced on average 410 kg less milk than single-calving cows.

However, contradicting results were found in another study [30]. Examining a herd of 23,588 cows of a local breed improved with Holstein Friesian between the years 2000 and 2008, it was

found that the average milk yield in kg after calving was found to be 6219 for single-calving cows, 6434 for twin-calving cows, and 7097 for cows calving triplets.

In a study performed in the USA [41], the effects of twin calving on the lactation in Holsteins were studied from a compilation of calving records from the Eastern Artificial Insemination Cooperative. The records of cows were grouped according to whether or not the twin calving was associated with dystocia. Records of each twin-calving cow were paired with records of a single-calving control herdmate. In the twin group of 175 cows that had difficult calvings, twin pregnancy was not found to have an effect on the cows' production; milk and fat yield in the lactation initiated by twinning were depressed; however, this was not significant. In the group of 367 twin-calving cows with no dystocia at calving, milk and fat production were depressed by 285 and 14 kg in the lactation after the calving. It was shown that twinning associated with dystocia seemed to depress the milk yield in the subsequent lactation (year 2). Compared to controls, twin-calving cows outperformed their herdmates in both the lactation before the twin calving (year 1) and the lactation after the next successful calving (year 3). Separate analysis of the 172 individuals with milk records for years 1 and 2 gave mean yields for year 1 of 7848 kg for twin-calving cows and 7722 kg for the control cows. For year 2, the mean for twin-calving cows was 7794 kg compared to a mean of 7811 kg for control cows. Although these means also indicate a depressive effect of twinning, this interaction was not considered significant. Mean values for cows where twinning was not associated with dystocia showed that twinning also depressed subsequent milk yield. The twin calvers outperformed their herdmates in years 1 and 3. No depression was noted for milk yield in the twin pregnancy lactation (year 1). There was also significant depression in fat percentage with twinning combined with a depression in milk yield to depress fat yield. In a group of 305 pairs in which no dystocia with twinning was observed, fat yield also was depressed in the twin pregnancy lactation. Twin calvers outproduced their herdmates in year 3, the year not directly affected by a twin calving. Both groups showed roughly the same effect of twinning on the number of open days following a twinning (**Table 2**). The 100 twin calvers associated with dystocia showed a noticeable increase in the number of open days following twinning. The 157 twin-calving cows with records for years 2 and 3 were open for an average of 105 days prior to conception of twins and 132 days after birth of twins as compared to means for the controls of 101 and 106 days. Analysis of the conception rate of 212 pairs of cows in which twin calvers had no difficulty calving showed that conceptions with twins averaged 9 days earlier when compared to conceptions of single-calving herdmate controls. After the twin birth, twin calvers took 22 days longer to conceive than their herdmates. When 316 pairs were examined for years 2 and 3 only, twin calvers showed a mean number of days open prior to conception of twins of 100 days and a mean of 131 days to conception following the twinning as compared to means of the controls of 105 and 108 days.

7. Consequences of twinning

Twinning in dairy cattle can have both favorable and unfavorable results, but it is not seen as a desirable trait in dairy cattle. Delayed rebreeding and other reproductive problems in twinning cows are some of the reasons for culling by farmers, whereas cows calving singles are culled more due to low milk production [26]. Twinning in the dairy herd population appears

to be increasing over time. Cows having twins are at greater risk for many periparturient reproductive and metabolic disorders than cows having single calves. Abortion, stillbirth, neonatal calf mortality, and reduced birth weights are seen more with the birth of twins than with that of single calves [30].

7.1. Gestation length

It was found in a study [27] that the average length of gestation before calving on the studied farm was 276.3 days. Here we experienced significant ($p < 0.001$) difference of approximately 5 days by type of calving. Twin-calving cows' gestation length (274 days) has proven to be shorter than that of nontwinning (278.7 days) herdmates. As their gestation is shorter and their reproductive performances are better, with shorter regeneration time and higher conception rates, they have a short calving interval. However, the benefits of this shortened calving interval before twinning are negated by the reproductive problems following twinning. There are more factors influencing the profitability of dairy cows than calving interval alone.

7.2. Dystocia and perinatal mortality

Dystocia means a difficult or abnormal delivery that occurs more frequently with twin births than with single births. In any calving, dystocia can be due to fetopelvic disproportion, an oversized calf or a dam with incomplete development or incomplete cervical dilation or due to abnormal presentation of the calf. However, twin calvings have the added complication of both calves entering the birth canal at the same time. These are usually dealt with by some sort of intervention.

Multiple births can cause difficulties at parturition, if parts of more than one fetus enter the birth canal at the same time [42–48]. These abnormalities can occur when two of the fetuses are released from the uterine horns at the same time and meet in front of the pelvis [49].

The symptoms of dystocia are highly variable, since each of the fetuses may be presenting in the anterior or posterior positions with variable positioning of the head and legs [50]. Therefore, these presentation anomalies occur more regularly in twin births than in single births [28, 42, 43, 45, 46, 51–53].

The occurrence of posterior presentation in twin birth is approximately four times higher (10.7%) than in single births (2.5%) [54]. Abnormal presentation was the cause of dystocia in 15.3% of the singletons and 77.8% of the twins. However, this can be easily corrected in twins due to their relatively low body weight, but nevertheless these factors still can result in stillbirths when there is a lack of observation during parturition. Obstetrical intervention was implemented in 42.2% of twin births and 20.4% of single births [28].

Some studies in the USA have demonstrated that the incidence of calving difficulty has decreased within the last number of years [26], whereas conflicting studies have shown the opposite, that it has not changed [55]. In this period of time, a gradual increase of twinning and periparturient mortality was detected [26, 55].

An analysis of the relationship between multiple births and dystocia [56] data was drawn from the SYMLEK database and provided information on 148,385 calvings of Polish Black-and-White

Holstein Friesian cows. The animals used for the study first calved in 2006 and were in use or had been culled by the end of 2012. All the data were classified in accordance with the SYMLEK database. The calving ease was classified as unassisted (natural, without human intervention), easy, difficult (using much more force than normal), very difficult (surgical procedure, injury to the cow or calf, embryotomy), or cesarean section. Calf mortality was classified as: live calf (normal live calf born), dead calf (stillborn calf or calf having died within 24 hours after birth), and the perinatal mortality. The goal is to investigate the relationship between the number of fetuses and gender of the fetuses on the outcome of the birth. The study found in the analyzed population there were 2.11% twin pregnancies and just 0.01% triplet pregnancies of the calves born. The proportion of unassisted parturitions was around 31% for single and twin births and only 7% when triplets were born. The proportion of difficult births increased from 4.3 to 28.6% with the increasing number of fetuses. Very difficult births and cesarean sections were sporadic and occurred with similar frequency when single calves and twins were born. For single calvings out of a total number of 145,241 calvings, 31.14% were unassisted, 64.35% were easy, 4.30% were difficult, 0.15% were very difficult, and 0.05% required a cesarean section. For twin calvings out of a total number of 3130 calvings, 30.96% were unassisted, 63.67% were easy, 5.21% were difficult, 0.10% were very difficult, and 0.06% required a cesarean section. For triplet births out of a total number of 14, 7.14% were unassisted, 64.29% were easy, and 28.57 were difficult with no births described as very difficult or requiring a cesarean section. Analyzing the gender ratio, most single calves were bull calves. In twin calvings, opposite sex twins were the most common, with bull calves being the least frequent. Opposite-sex calves were most common among triplets.

The mortality rate is four times higher in twin-born calves due to an increased dystocia and a reduced gestation length; furthermore, twin calves had a lower birth weight [6].

Perinatal mortality is also a major factor related to parturition. Twinning-associated perinatal mortality was three times higher than that of pluriparous [51]. However, the number of calves born alive was higher among mothers of twins and triplets compared to mothers of single-born calves [14].

Analysis of the results (**Table 4**) shows that heifer calves tend to be born more easily than bull calves; however, this relationship only was significant in single births ($P \leq 0.01$). In twin births, when two heifers were born, they tended to be easier, whereas difficult births, very difficult births, and cesarean sections tended to be more frequent when a bull and a heifer calf were born. The results show a higher perinatal mortality in twins; however, even including the losses, on average 1.81 live calves were produced from twin calvings compared to only 0.92 from single calvings. When analyzing calf mortality's relationship to gender, in single calvings, the number of bulls and heifers born live was similar (46%). However, the percentage of stillborn bull calves was over 3 times that of heifer calves. Examination of twin births showed that the proportion of both calves being live-born was highest (39.84%) when twins were of opposite sex and lowest (22.81%) when twin bulls were born. Perinatal mortality tended to be highest (3.19%) when twin bull calves were born and over twice as low when twin heifer calves were born. When opposite gender twins were born, perinatal mortality was 2.65%, and

Type of birth	Sex of calf	Parturition					
		n	Unassisted	Easy	Difficult	Very difficult	Cesarean section
Single	Heifer	69,789	34.9	61.88	3.12	0.09	0.02
$\chi^2 = 1243.78$ $p < 0.01$	Bull	75,452	27.67	66.63	5.4	0.22	0.09
Twin	Heifer + heifer	872	28.78	67.2	3.9	0.11	0
$\chi^2 = 13.54$	Bull + bull	841	30.68	64.09	5.23	0	0
	Heifer + bull	1417	32.46	71.26	6	0.14	0.14
Triplet	3 Heifers	3	0	33.33	66.67	0	0
$\chi^2 = 3.61$	3 Bulls	3	0	66.67	33.33	0	0
	Bulls + heifers	8	12.5	75.00	12.5	0	0

Table 4. Calving difficulty by number and gender of calves born [56].

when one of the calves was alive and the other was dead, heifers tended to survive more often. The proportionally small number of triplet calvings (only 14 cases) and the various gender combinations in triplets made the results difficult to interpret. Examination of the results confirms that bull calves were more likely to die perinatally than heifers, and multiple pregnancies increase calves' risk of perinatal mortality. The increased calving difficulty associated with multiple births majorly increases the risk of perinatal mortality in calves. In calvings classed as very difficult, the risk of calves being born dead or dying within 24 hours after birth increased 10-fold, independent of gender, in relation to unassisted calving.

Twin pregnancies have a higher risk of fetal death or abortion in the final months of gestation [57, 58]. The limited energy reserves and vitality of twin calves during and after pregnancy are seen as the primary reasons for the elevated proportion of stillbirths [59, 60]. The shorter intrauterine period is also thought to be a reason for the lower birth weight.

This is preventable by supplying a targeted diet with adequate nutrients to the dam in late pregnancy and also drying cows off early when twins are expected. The rate of stillbirth in twin calves was 19.0% and 12.9% and in single calves 5.0% and 4.1% [5, 28, 61]. Two different types of embryonic death can be distinguished [18]: "independent loss" describes the phase prior to implantation and in this phase, losing one or more embryos does not affect the rest, whereas in "dependent loss" the embryo dies after implantation, causing a loss of all other embryos due to the placental anastomosis.

At parturition, twin calves are at a higher risk of hypoxia if the umbilical cord becomes entangled in a leg of the other fetus [62].

7.3. Milk yield

A study in America [63] showed the effect of twin-birth calving on milk production. Primiparous and multiparous cows with singletons produced more milk than cows with live twins or at least 1 dead twin (primiparous, 33.1 vs. 31.9 vs. 31.2 l; multiparous, 36.5 vs. 35.7 vs. 35.0 l,

respectively), which was in good concordance with results of another study [40]. In contrary to these reports, mothers of twins have been shown to be superior to mothers of single calves in terms of milk yield in a third investigation [30].

7.4. Fertility and reproduction

The reproductive health of the herd is a great indicator of how a management system is working, by highlighting the profit or losses seen financially. With dairy cattle, these results can be seen from the view point of reproduction (calving interval, dry period, and services per conception) and milk production (milk yield and fat and protein %).

Ideally, every farmer would like his herd increase every year, with each cow having at least one calf, and in turn increase the milk yield with every subsequent lactation. In reality, multiple births come with unwanted problems. Unfortunately, more calves per cow do not automatically mean more profits seen.

From higher incidence of abortions, reduced birth weight or higher numbers of stillbirths, mastitis, and problems related to dystocia, the increase in costs in the case of multiple births adds up to 40% per cow [64].

There is a higher incidence of pregnancy loss, with number of fertile heifers' herd replacement [15].

Increase of calf mortality is 18% [30] and fertility of the cows was shown to deteriorate after multiple pregnancies, with the most unfavorable indicators of fertility found in triplet births [14].

Other studies show that twinning increases open days and services per conception on average and therefore decreases reproductive performance of the dam during subsequent lactations [21].

There is a longer regeneration time of the uterus and this in turn causes the elongation of the resting period after calving-these were increasing with the number of lactations, relating to twin calving and shorter gestation. This was more evident with calving in the summer period.

Cows with dead twins also had increased time to conception, compared with live twins. The natural incidence of multiple births in cattle is largely due to multiple ovulations and is around 1–5%, depending on breed, parity, and environmental conditions [65].

Analysis of bovine pregnancy–associated glycoprotein (bPAG-1) can also be used to determine the nature of the pregnancy from about day 90 [66]. Dissemination of twin and singleton pregnancies is not possible before day 85, so prevention or early identification of twinning is not possible with this measurement. To achieve diagnostic test with both high sensitivity and specificity, further studies such as ultrasound are required.

7.5. Freemartinism

The double ovulation occurs when two mature follicles are released at the same time in one cycle of ovulation [21]. Adhesion of the outer layers of the amniotic sacs can occur in twin and multiple pregnancies because of their proximity. This may result in fusion of the chorion layers at which point an anastomosis of the vessels occurs in most cases leading to a union of the blood circulations of both embryo. In case of twins with different sex (bull and heifer), in 98%

of all occurrences, heifers are not suitable for breeding because of freemartinism [67], causing infertility in the female calf born with a male twin. As fertile heifer calves are far more valuable than bull calves, this is an issue on dairy farms.

7.6. Culling reasons and life span

It has been shown [14] that multiple pregnancies considerably decreased the chance of survival until the next calving and increased the culling rate. When analyzing the reasons for culling cows from the herds, it was found that multiple births gave rise to a greater proportion of cows culled due to udder diseases, infertility, reproductive diseases, old age, metabolic and gastrointestinal diseases, and diseases of the locomotor system.

The lifespan of 3581 cows that had never had twins and 386 cows that had twins at least once was examined [38]. On average, the total lifespan was 68 months. Whether or not the cows had twins had a significant impact on their lifespan ($p < 0.001$). The cows that never had twins reached on average 60 months (roughly 5 and a half years old), and cows that had at least one set of twins reached on average 76 months old (roughly 6 and a half years). The difference in life expectancy between these two groups was 15.8 months (a year and 3 months approximately).

7.7. Economic outcome and selection

Losses due to the higher incidence of abortions, ketosis (subclinical or clinical), reduced birth weight or stillbirths, mastitis, and problems related to dystocia are all more common in twin calvings. Multiparous cows with dead twins produced less milk than cows with live twins. Compared with dams with singleton birth, cows with twins were 0.78 times as likely to conceive and 1.42 times as likely to die or be culled. Cows with dead twins also had increased time to conception, compared with live twins [63].

This increased cost in the case of multiple births adds up to 40% per cow [64]. In the case of twinning, there is an elevated incidence of pregnancy loss and reduced milk yield along with the number of fertile heifers' required for herd replacement [15].

Total losses were on average \$171 – \$63 = \$108 per twin birth. Realistic changes in input variables could not change this negative outcome to a positive result. Therefore, it was concluded that it is not profitable to select to increase the number of twins in dairy cattle [68].

Dairy cattle breeders could develop strategies to manage twinning based, for example, on ultrasonic examination of corpus luteum and its direct use in selection. While it is obvious when cows give birth to twins, it is much less obvious when cows that would have twins suffer embryonic reduction of one of their embryos. An examination [69] of 770 pregnancies showed 13 cows with 3 or more corpora lutea and 757 with 2 corpora lutea. Of those with two corpora lutea, 464 were carrying twins and 293 were carrying single calves. Subsequently, 69 (23.5%) of the single pregnancies and 132 (28.4%) of the twin pregnancies lost one of the corpora lutea or one of the embryos before day 60. Of the 132 twin pregnancies, 34 (25.8%) lost a corpus luteum together with an embryo (corpus luteum reduction occurred in the ovary on the same side as the horn of the uterus that underwent embryo reduction). As dead embryos and their debris

are not detectable when they die in early pregnancy, this represents a "hidden rate of twinning" that we have otherwise overlooked.

There is an opportunity to change the incidence of twinning in Holstein cattle when the candidate bulls are provided with a breeding value for twinning rate [70]. With heritability at 8.71%, genetic evaluation of sires is possible [26]. Centered about a mean twinning rate of 5.02%, PTA of sires ranged from 1.6 to 8.0%. Therefore, use of sires with a low PTA for twinning rate can be expected to reduce the incidence of twins. Some increase in income can also be expected with a reduced incidence of twins [71]. From a national perspective, this translates into a cost of $55 million per year to the dairy industry in the United States, assuming 5% incidence of twins, 10 million dairy cows in the United States, and $110 less income per head.

Trying to make a profit from increasing the incidence of twinning within a herd would be very difficult due to the time and money increase associated with twin births. The benefits of twinning cannot be capitalized on without some degree of cost, either financial or reproductive. This can be mainly seen at parturition and postpartum because of the complications associated with twin births.

8. Conclusion

From all of the studies on the subject, it can only be concluded that the occurrence of twin calving is a double-edged sword, as it is a multifaceted trait with many pros and cons that cannot easily be reconciled. On the one hand, we see the benefits of higher numbers of live calves, and on the other hand, we see decreased fertility in cows after twin calving, leading to higher culling rates and thereby decreasing the total number of calves over the lifespan of the cow. That and the decrease in milk production are clearly very negative traits to have in dairy cows.

However, we see cows that have the highest production value in the herd are predisposed to twinning, and increasing nutritional intake to improve productivity also increases twinning. Obviously, it is in the farmer's best interest to breed and feed his cattle as well as possible to increase productivity and profits. Leading to an increase in the rate of twinning and all the negative traits associated with it.

Decreasing the rate of twinning by using bulls with a low PTA for twinning shows some promise as a method of avoiding all of its negative consequences; however, there are many traits in bulls that farmers may value more such as milk solids or milk yields and so may not be practical to choose bulls based on this.

It is hard to say what the best course of action is for dealing with twin calving. But it is advantageous to be aware of its consequences so farmers can deal with them to the best of their abilities.

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Animal Health

Chapter 5

Dairy Cows Health Risk: Mycotoxins

Violeta-Elena Simion

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.72709

Abstract

Mycotoxins are secondary metabolites of mycotoxigenic fungi affecting both human and animal health. Their production in plants is highly unpredictable and dependent on a variety of factors, as well as the stage of the culture and transportation, storage and processing of the raw materials. One of the risks for dairy producers is animal exposure to mycotoxins. The scientific literature shows nonspecific signs to appear in a herd, most often when mycotoxins are present in feed and worse, in milk. In general, ruminants are considered resistant to the action of most mycotoxins, attitude explained by the detoxifying role of ruminal microsymbionts and especially protozoa. The clinical examination performed on the dairy cows from the studied farm did not reveal the presence of any symptom characteristic to mycoses or to mycotoxicosis. Although considered resistant to the action of mycotoxins, research reveals the constant presence of mycotoxigenic fungi and the mycotoxins they produce in the fodder of dairy cows, many times in various combinations. The incidence of mycotoxins is unpredictable and influenced by numerous factors (climatic, of production, transport, processing and storage of fodder). The health of dairy cows is affected by the consumption of contaminated fodder.

Keywords: fungus, mycotoxins, dairy cows, milk, health

1. Introduction

The notion of fungus refers to a large array of eukaryotic structures, unicellular or multicellular, as they are regulated through ISO 21527–1 and 21,527–2, which cancel and replace ISO 7698:1990, ISO 7954:1987 and ISO 13681:1995.

Molds are free of chlorophyll, mesophilic aerobic filamentous microorganisms which, on the surface of mycological agar medium, under some conditions (0.7–0.9 water activity (a_w) and usually 25 ± 1°C temperature), develop flat or fluffy spreading propagules/germs or colonies often with colored fruiting or sporing structures [1, 2].

The majority of microfungi are saprobiotic organisms that can be seen in all-natural media – earth, water, air. In nature, fungi can grow and invade any type of food, at any moment, if the conditions favorable for their growth are created. The latter are in general represented by: substrate humidity higher than 11.5%, relative humidity of the air of over 70%, oxygen presence of 1–2%, temperatures between 5 and 40°C, substratum pH of 4–8, a_w between 0.7 and 0.85, relatively low light intensity and plant stress under the action of unfavorable medium factors (action of damaging insects, climate factors etc.).

The direct action of fungi over live organisms is of tissular destructive levels and can be limited or generalized, determining diseases named mycoses. In the pathology of ruminants, the following types of fungi are generally involved: *Absidia, Alternaria, Aspergillus, Candida, Cryptococcus, Fusarium, Mucor, Penicillium, Rhizopus, Rhodotorula, Sporothrix, Stachybotrys, Trichoderma, Trichophyton, Trichosporon*. Of these, there are certain types which are recognized as having mycotoxigen potential: *Aspergillus, Fusarium, Penicillium, Mucor* and *Rhizopus*. According to FAO/IAEA, mycotoxines are secondary metabolites of fungi, non-volatile, organic, developed by fungi in both food and fodder [3].

The optimal temperature and humidity for developing a species of fungi does not correspond to the optimal parameters mentioned to produce mycotoxins, which determines a disparity between the presence of a species of fungi and the mycotoxins developed at that moment in the respective substrate. Note that there can be situations in which we find in analyzed food or fodder either only fungi, fungi and the mycotoxin/mycotoxins they produce or only mycotoxins. Mycotoxins are developed through a secondary metabolic process, which differs to the primary metabolism through its random nature, the diversity of compounds developed and the specificity of the thalli involved. The metabolic chains involved in the production of mycotoxins respond to the signals received by the fungus from the outside medium, thus not being related to cellular growth.

The diseases resulting from the activity of mycotoxins are named mycotoxicoses. The acute forms have a rapid evolution and are produced due to the action of high doses of mycotoxin over an organism. The chronicle forms, much more common, imply a slow development of the infection.

In general, ruminants, compared to monogastric animals, are considered resistant to the action of most mycotoxins, attitude explained by the detoxifying role of ruminal microsymbionts and especially protozoa. Ruminal and intestinal microorganisms do not significantly degrade mycotoxins when the ruminant's food is rich in concentrated fodder, as an example, or when the quantity of mycotoxins ingested reaches over certain limits. Equally, rumen metabolites of the parent mycotoxins can become, after ruminal biodegradation, not just less toxic but, in some cases, also more aggressive than the initial substance. Even so, the clinical examination performed on the dairy cows from the studied farm did not reveal the presence of any symptom characteristic to mycoses or to mycotoxicoses at dairy cows. From this perspective, it is extremely useful the analysis of the quality of fodder in regards to their contamination with fungi and/or mycotoxins and the application of preventive measures for the health of the animals.

2. Mycotoxin occurrence and mycotoxicosis

Mycotoxins are secondary metabolites developed under increased temperature, humidity, a_w, pH and their presence in fodder cannot be detected organoleptically. Furthermore, the

differences in the conditions of production for fungi and the mycotoxins associated to them are significantly different.

In general, the favoring factors for the development of fungi and the production of mycotoxins can be divided into three main categories:

- Physical: relative humidity, substrata humidity, water activity, temperature, fodder integrity.
- Chemical: pH, substrata composition of nutritive substances.
- Biological: presence of some microorganisms and/or invertebrates.

In general, with a_w of 0.85 at 25°C which corresponds to approximately 14–16% humidity, fungi spores germinated within 5 to 12 days.

Moreover, the effects of mycotoxins consist of:

- The reduction of the ingestion of food until it is completely refused y the animal.
- Reduction in the absorption of nutrients and the affliction of the metabolism.
- Alteration of the endocrine and exocrine systems.
- Suppression of the immune system.
- Reduction in the reproductive performances until the entire reproductive system is affected.

The symptoms are most often nonspecific, which makes the diagnostic difficult or even impossible. The difficulty in diagnosing mycotoxicoses is given as well by the occurrence of multiple mycotoxins, their uneven distribution in the fodder mass, the influence of certain factors linked to the animal, ration and climatic conditions.

2.1. Aflatoxins

Aflatoxins (AF) are mycotoxines produces in nature by fungi species of the *Aspergillus* (*A. flavus* and *A. parasiticus*) and more rarely *Penicillium* (*P. puberulum*, *P. citrinum*, *P. variable* and *Rhizopus* types. The notion of aflatoxin in common languages refers to all its four representative forms: AFB_1 ($C_{17}H_{12}O_6$), AFB_2 ($C_{17}H_{14}O_6$), AFG_1 ($C_{17}H_{12}O_7$) and AFG_2 ($C_{17}H_{14}O_6$). *A. flavus* produces AFG_1, AFG_2 as well as AFB_1 and AFB_2. Aflatoxins B_2 and G_2 are dehydrogenated derivatives of AFB_1 and AFB_2. From a mycologic point of view there is a large quantitative and qualitative difference regarding the ability of the different fungi species of producing these mycotoxins, about half of the *A. flavus* species producing aflatoxins [4].

The AF group comprises around 20 mycotoxins (e.g. M_1, M_2, B2a, AFL, AFL-M_1, P_1, Q_1, H_1 etc.). The M_1 and M_2 metabolites are hydroxylated derivatives of aflatoxins B_1 and B_2 secreted in the animal milk following the consumption by the cows of food that has been contaminated with these [5].

At present, the kinetics of the transformations and the risk associated with the consumption and absorption of aflatoxins is well known, both at animals and at humans. The main conjugation way of aflatoxins is glucurono-conjugation, the resulting complex being eliminated

through the bile. The liver is considered the target organ for aflatoxins. At this level, the metabolization of mycotoxins takes place under the action of microsomal enzymes. The reaction products are eliminated from the organism through excretion products (feces and urine) and milk in unmodified form as well as metabolites.

The studies regarding the distribution and metabolism of AFB_1 marked with C^{14}, in the organism of some animals, have demonstrated both the elimination in significant quantities of the mycotoxin from the organism in the first 24 hours, as well as the accumulation of the residual quantity in different organs (muscles, stomach, liver, heart etc.), accumulation conditioned by the dosage of mycotoxin ingested.

Naturally, ruminants seem to be more resistant to the action of aflatoxins compared to other species of animals, although the clinical signs of aflatoxicosis have been observed in cows, such as: the reduction in the ingestion of food, the decrease in the production of milk, the affliction of the hepatic function. The chronic exposure to the ingestion of aflatoxin determines an inefficient feeding, depression of the immune system and the reduction of the reproductive function [6].

The lipophilic mycotoxins with a small molecular mass like AFB_1 are absorbed in the digestive tract through passive diffusion. Aflatoxins, like other mycotoxins, induce severe hepatic dysfunction confirmed through biochemical tests in numerous studies [7, 8].

The pathologic modifications are more alarming in dairy cows, with high production, which are more sensitive to toxins. AFB_1 is a strong inhibitor of the protein synthesis which blocks *in vivo* the replication and transcription of DNA and inhibits the synthesis of RNA and proteins. The metabolic products of aflatoxin act on the chromatin inhibiting the transcription of genes and RNA polymerase, which as a result produces a decrease in the concentration of RNA and protein synthesis. *In vitro*, AFB_1 effectively couples with the DNA and causes irreversible mutations, which explains its incredibly strong carcinogenic effect. Approximately 90% of AFB_1 is present in blood, in plasma, being linked especially to albumins. Aflatoxins are oxidized in the liver with formation of very reactive molecules, capable of binding the nucleic acids or functional proteins. This hepatic bioactivation has a considerable importance for animal health due to the active metabolites that form *in situ*, at tissue level. The metabolization of aflatoxins at hepatic level takes place under the action of the microsomal enzymes, the most active of these being P450.

After the oral administration of AFB_1, the metabolites are quickly found in urine and milk, while small quantities can be distinguished in feces which confirms the rapid absorption of AFB_1 in the digestive tract and hepatic metabolism [9].

In general, the ruminal degradability of AFB_1 is minor and the toxicity of the metabolic products is similar to that of the parent molecule. Aflatoxins affect the ruminal function through the reduction of ruminal motility, the capacity of digesting celluloses, of producing volatile fat acids and proteolysis [10].

The ruminal juice and, moreover, the bacteria population from the cow and sheep rumen does not have the capacity to convert aflatoxins in other metabolites except for AFM_1 which is found in large quantities in milk [11]. Auerbach et al. have observed that adding 9.5 ng AFB_1/ml ruminal liquid did not alter *in vitro* the digestion of alfalfa and did not influence the production of volatile fat acids while, in another study, adding a dose of 1 μg AFB_1/ml highlighted the lowering of the ruminal capacity of producing the acids [12].

Moreover, *in vivo* studies showed the presence of AFM_1 in the ruminal content, which leads to the conclusion that AFM_1 produced in the liver can reach the rumen, through the rumeno-hepatic way [13]. At dairy cows, from the total of 4.52% aflatoxins detected in the organism, 1.55% was detected in urine, 2.79% in feces and 0.18% in milk, in the form of AFM_1 which represented 0.35% of the administered dose. At the sheep in lactation, from the 8.1% compared to the ingested dose, it was detected 6.4% in urine, 1.63% in feces and 0.1% in milk. After a period of 6 days from the administration, the aflatoxin was not detected anymore in milk, after 8 days in urine and after 9 days in feces [14].

The degradation of aflatoxins (AFB_1, AFB_2, AFG_1, AFG_2) in the rumen is relatively reduced, with a proportion of under 10% at a quantity of ingested mycotoxin of 1–10 μg/ml which is less toxic [15].

AFB_1 is considered the most carcinogenic natural substance being recognized as the most aggressive mycotoxin, for all the animal species, including human. The carcinogenicity of aflatoxins is dependent upon: animal species, age, dosage ingested, duration of ingesting the mycotoxin and nutritional state [16].

2.1.1. Aflatoxins and milk production

The transformation of AFB_1 from fodder in AFM_1 in milk is realized following a process of hydroxylation. AFM_1 and AFM_2 are metabolites (hydroxylated forms) of AFB_1 and AFB_2 found in milk. Other metabolites identified in cow milk are: AFM_4, AFQ_1 and aflatoxicol [17].

At dairy cows, the absorption of AFB_1 in the digestive tract is rapid and complete, which explains its almost immediate transfer in the milk, under the shape of AFM_1 [9]. Although many researchers have concentrated their attention on the study of AFM_1 from public health reasons, its production represents between 1 and 3% of ingested AFB_1, with an average of 1.7% [18, 19]. Other studies have reached the conclusion that certain quantities of AFB_1 in the food of dairy cows (13 mg impure AF/day, over 7 days) can induce the decrease in production, without the evident clinical sign of disease. The transfer rate of AFB_1 from fodder to milk has been variable, with values comprised between 0.3 and 2.2% [20]. Milk production and body weight returned to normal limits within the next 5–8 days from removing the contaminated fodder.

For preventing the risk of transmitting AFB_1 to the milk, the superior limit for the content of this mycotoxin in the fodder of dairy cows with a production of 20 kg milk/day, after ingesting 6 kg of contaminated fodder/day has been evaluated at 5 μg AFB_1/kg [21].

It was administered to a lot of lactating cows, in their food ration, corn contaminated with 120 μg AF/kg fodder. As a result, it was discovered the apparition of reproductive disorders, health problems, as well as the decrease in milk production. After removing the contaminated corn from the ration, the milk production rose to 28% within 3 weeks [22].

Diaz et al. [9] affirm that AF appear in milk after approximately 12 hours from the oral administration of AFB_1, the maximum quantity being registered after 24 hours from ingesting the aflatoxin.

The mathematical relationship between the ingested quantity of AFB_1 and the quantity found in the milk is:

$$AFM_1 \text{ (ng/kg milk)} = 10,95 + 0,787\ X \quad (1)$$

where X = μg AFB_1 ingested/day.

Similar studies have shown approximately 90% of AFM_1 present in the blood can be determined in the plasma and is found afterwards in the milk and urine [23].

The FDA limits aflatoxins to no more than 20 ppb in lactating dairy feeds and to 0.5 ppb in milk; in Europe, the regulatory levels of AFB_1 are 20 ppb for dairy feeds and 0.05 ppb in milk.

2.1.2. Aflatoxicosis

The aflatoxicosis is an acute or chronicle mycotoxicosis of mammals, as well as birds, produced by aflatoxins.

Etiologic agent, toxicity. Aflatoxicosis is induced by the aflatoxins produced by some species of fungi such as *Aspergillus flavus, A. parasiticus, A. niger, A. ochraceus, Penicillium puberulum, P. citrinum* and *Rhizopus spp.* From these, the main aflatoxin producers are *A. flavus* and *A. parasiticus*. Mycotoxins, as well as their metabolic products, work on the act on the chromatin, inhibiting the synthesis of the nucleic acids DNA and RNA from the hepatic cells and, at the same time, on the cellular membranes and membranes of the different intracytoplasmic structures.

Clinical signs. Aflatoxins produce disorders at the level of the central nervous system, digestive system, cardiovascular system and hematopoietic organs.

Aflatoxicosis evolves under acute, sub-acute and chronic form.

In acute form, it can be observed clinically: inappetence, anorexia, nervous depression, ataxia, dyspnea, and melena, anemia, while in sub-acute form: hemorrhagic enteritis, subcutaneous hematoma and jaundice.

In chronicle form, it can be observed: reduction of growth, rough and pale hair coat, anemia, jaundice, slower growth, apathy, diminished appetite, teeth grinding, fixed stare, circular movement, ataxia, diarrhea, rectal prolapse at mammals [24]; reduced resistance to diseases and interferes with vaccine induced immunity in livestock [25].

The toxic effects depend on: the dosage of mycotoxin ingested, the way of ingestion/administration, duration of exposure, nutritive quality of the ration, species and animal age. The calves, fresh cows or dairy cows in early lactation are most affected because their immune system is suppressed. Alongside *Aspergillius flavus, A. parasiticus*, producers of aflatoxin and *Aspergillius fumigatus* producer of tremorgens or viritoxin, fumagillin, encountered both in fibrous fodder, as well as silos, is considered as pathogen agent, being associated with the Mycotic Hemorrhagic Bowel Syndrome (HBS) at dairy cows [26].

Adult taurines and sheep are the least sensitive to the action of the mycotoxin. It is considered though that with the ingestion of at least 100 ppb aflatoxin/day, ruminants (both meat and dairy cattle) are exposed to the risk of the development of aflatoxicosis which debuts with: the reduction of food ingestion, diminished live weight, lowering of milk production, decline of the efficiency of the reproductive capacity [27, 28].

Anatomopathologic modifications. At the anatomopathological exam it can be observed: jaundice, generalized hemorrhage, hemorrhagic gastroenteritis, intestinal ulcers, hepatic necrosis, steatosis, hepatic fibrosis, hepatomegaly, cirrhosis, ascites, hydrothorax, thickening of the bladder walls and gall bladders [29].

Histological modifications. At the histologic exam, we can see perineural hemorrhage, perirenal edema, pioencephalitis with eosinophilic infiltrations and, in the case when it crosses the placental barrier, AF produces hepatic and digestive micro-lesions starting from the intrauterine life [24].

The most important action of the aflatoxins is considered to be the carcinogenic one, as well as the accusation of the preexisting tumoral formations. Neoplastic lesions are produced especially at hepatic level, but also at the level of the digestive tube, with lungs, kidneys metastases etc. Neathery et al. have observed that, in the situation when food is administered containing AFB_1 (0–5 ppm) and zinc (40–640 ppm), at calves, they showed some of the characteristic clinical signs of aflatoxicosis: reduction of food ingestion, lowering of live weight, reduction in pulse values and respiratory rhythm; no other anatomopathological modifications were seen in the liver or other organs [30].

Presumptive diagnosis is established based on the epidemiologic enquiry, clinical examination and anatomopathologic exam, while the certainty diagnosis is done on the basis of paraclinical examination (drop in vitamin D in the liver and the increase in alkaline phosphatase with return to normal before death) and of mycologic and mycotoxicologic examination of the fodder.

2.2. Ochratoxins

Ochratoxins are compounds developed by different species of *Penicillium* (*P. viridicatum, P. commune, P. purpurescens)* and *Aspergillus* (*A. ochraceus, A. alliaceus, A. melleus,* etc.) which contaminate cereal grain, vegetables and combined forage.

Ochratoxins A (OTA), B (OTB), C (OTC), D are a group of compounds in whose chemical structure L-fenilalanin is coupled through an amidic link with an isocumarinic derivative. The production of these mycotoxins is frequent in molded or overheated fodder and is favored by the presence of oligoelements, a temperature of 20–28°C and a humidity of 18–19% at wheat, in the case of *P. viridicatum* or 22% in corn. In laboratory conditions, most strains of *A, ochraceus* produce OTB and OTC, while in natural environment conditions, the most frequent is OTA. Its precursors can also be seen in culture media [23].

OTA is passively absorbed, in unionized form, at the digestive tube level, especially at the level of the short intestine level, at a pH of 7.04. After OTA's penetration of the organism, the mycotoxin links itself to the albumines in the plasma and starts rapidly metabolizing, depending on the animal species. Among the organs, OTA's highest affinity is for the liver and kidney. OTA has the strongest inhibitor effect on animal growth, determining the excessive accumulation of glycogen in the liver of afflicted animals [31].

The halving time of OTA in studies on monkeys was 840 hours, or several days in the case of pigs [32] and 3 hours in chicken [23]. The most important transformation takes place at hepatic level, where OTA is metabolized through hydroxylation in 4-hydroochratoxin A, metabolite eliminated by the kidney [31, 32].

At the level of the digestive tube, where afterwards it is also absorbed, another important metabolite is formed, OT-α, which is finally eliminated through the digestive system or the kidneys. Other metabolites formed in the organism follow an enterohepatic circuit after which they are also eliminated through feces and urine [32].

Höhler et al. affirm that sheep fed with fodder containing various concentrations of OTA multiple values were recorded. Thus, at 2 ppm added OTA in food, there were significant concentration of OTA and OTA-α in the sanguine serum (10 and 3 ng/ml) and much higher concentrations for both mycotoxines at 5 ppm added OTA in food (80 and 15 ng/ml) [33].

The quantity of mycotoxin degraded by the intestinal and ruminal microsymbionts is dependent on the quantity of concentrates from food, the absorption of mycotoxins being higher at a larger content of starch in the food compared to fibers. From the total quantity of OTA ingested, approximately 70% of it is eliminated as OTA – α (9% in feces and 61% in urine) compared to the underrated OTA form (1% in feces and 3.8% in urine) [9]. Recent studies have shown the ability of OTA to disturb the cellular signal and to influence the viability and proliferation of cells [34].

OTA was determined in quantity of 2.2 μg/kg in the oat assay and in amount of 3.2 μg/kg in the bran assay. In the case of this mycotoxin, the results obtained revealed a quantity of 0.1 ng/ml in blood serum, 0.018 ng/ml in milk and 0.009 ng/ml in urine. Although it can be observed that from the 5.4 μg/kg ingested OTA, 1.8% were transferred in the blood serum, 0.3% in milk and 0.1% in urine, the conclusion regarding the conversion rate is uncertain, as different studies regarding the absorption and excretion of OTA and OTA-α at ruminants have shown the major influence of the type of food on the metabolites transfer to blood, milk and urine. The protozoa population at the rumen level is largely influenced by the type of alimentation of the ruminants. For example, the transformation of OTA in OTA- α is favored by feed rich in starch more than one rich in fibers [23, 35]. In a study on sheep, Blank et al. administered OTA through wheat contaminated with mycotoxins, at a base ration of 70% concentrated feed and 30% silo. The study showed that a large part of the OTA quantity remained undegraded and was detected in the sheep serum (from 1.5 to 18 μg OTA/kg BW/day), regardless of the OTA level in the food, while the quantity of mycotoxin excreted in the urine remained almost constant (6–8% of the ingested dose), regardless of the food dose. Alongside OTA, small OTA-α were detected in the serum (from 0.5 to 1.6 μg OTA-α/kg BW/day), directly proportional with the of the quantity of OTA in the food [36].

An important aspect of the metabolization of OTA in the organism is represented by the renal absorption at the level of the proximal tubes (2/3) and at the level of the distal tubes and collector channel (1/3). This phenomenon takes place due to the disturbance of pH homeostasis at cellular level of the nephron walls, which affects the acid–base transepithelial transport and determines the acidification of urine. The latter favors the reabsorption of OTA leading to the accumulation of the mycotoxin in the organism through the reduction of the elimination rate [37, 38].

Protozoa are considered organisms with a major role in degrading OTA to OTA-α. Other factors that can significantly influence the metabolic rate of OTA in the ruminant organism are: the animal age, genetic structure, health of the ruminal microsymbionts, alimentary ration structure.

The quantity of mycotoxin degraded by the intestinal and ruminal symbionts is dependent on the quantity of concentrates from the food, the absorption of mycotoxins being higher at a higher content of starch in the food compared to fibers. The inhibitor effect of OTA on the bacterial growth was only observed in the case of gram-positive bacteria, in general under a neutral or lower than 7 pH [9]. Müller estimated through *in vitro* studies that adult cows are capable of degrading 33 to 72 mg OTA/day, while sheep are capable of 3 up to 7 mg OTA/day. The food composition influences the structure of the ruminal microsymbionts and, implicitly, the capacity of degrading OTA. When the mycotoxin is found in a large quantity in the food, the microbial detoxification capacity is reduced and the symptoms of the ochratoxicosis appear. Moreover, the OTA metabolism in the rumen is much reduced in the case of the digestion of an increased quantity of concentrated fodder compared to an alimentation richer in fiber [39].

2.2.1. Ochratoxicosis

Due to the detoxifying capacity of ruminal symbionts for OTA, ruminants appear to be more resistant to the action of ochratoxins compared to monogastric animals. This capacity is the more evident the healthier the population of microsymbionts, especially protozoa. An alimentation rich in concentrated fodder affects the level of ruminal pH, consequently affecting the protozoa population and, implicitly, the capacity of metabolizing mycotoxins, in this case OTA. Moreover, after the administration of a dose similar to the one naturally found in fodder, OTA and OTA-α were not detected in milk, which is explained by the degradation of the ochratoxin in the rumen by the microsymbionts.

From the clinical signs of ochratoxicosis at ruminants, we distinguish the development of the pulmonary edema and the damaging of the animal health up to its death at OTA concentrations of over 3 ppm/kg in the fodder. At the same time, studies have shown that OTA does not cross the placenta barrier in the case of the oral administration of mycotoxin in reduced quantities (0.38 mg OTA/kg), although this was detected in the cow milk, as well as in ilk from other animals (pig, rabbit, rat).

2.3. Zearalenone

Zearalenone (ZEA) is a mycotoxin produced by fungi species of the *Fusarium* (*F. graminearum, F. tricictum*) and is most commonly found in cereal grain.

From a chemical point of view, zearalenone ($C_{18}H_{22}O_5$) is a lactone of the resorcilic acid, with a structure similar to steroid hormones [5].

An experimental study followed the administration of 385–1925 ppb ZEA during 7 weeks din not show a change in milk production, nor the presence of ZEA residues in milk, serum, urine or tissue. Both ZEA and its metabolites are absorbed at intestinal level, covering the enterohepatic cycle [40].

α and β-zearalenol are derivatives of ZEA that are eliminated from the organism through feces and urine and, to a lower degree, through milk. From these, α-zearalenol is considered the metabolite with the strongest estrogenic activity. Another metabolite of ZEA, which only develops in reduced quantities in animals, is zeranol, substance with a strong anabolic effect [41].

2.3.1. Toxic effects at ruminants

The symptoms of ZEA toxicosis are: uterus hypertropia, swelling of the vulva and mammary glands, decline in the ovulation rate and disturbance of the heat cycle, conception rate is low at dairy cows, the estrogen effect of ZEA being owed to the link of the mycotoxin to the cytoplasmic estrogen receptor.

It was discovered that the milk production decreased, infertility and hypoestrogenism appeared in the case of cows that consumed fodder contaminated with ZEA or with other fungi of the *Fusarium* type. Coppock et al. have shown that the effects of ZEA over the reproductive apparatus (vaginitis, vaginal secretions, mammary gland enlargement) at dairy cows can be strengthened through the synergic action of 600 ppb ZEA and 440 ppb DON in food; the consumption of food decreases which leads to the reduction of milk production, cases of diarrhea, increased infections of the reproductive tract and the entire reproductive activity is compromised. In general, it is considered that 400 ppb ZEA in the food is the maximum concentration for which the reproductive activity of dairy cows is not affected [42].

A secretory activity of the mammary gland was observed at heifers that consumed fungi contaminated corn in the pre-puberty period. The administration during three estral cycles, at a heifer lot, of 250 mg purified ZEA, determined the reduction of the conception rate with 62% while at the control lot, the rate of conception was reduced by 87% [43].

Signs of hyperestrogenism were shown in cows that consumed fodder contaminated with 1 mg ZEA/kg fodder, over 5 days, while at sheep that received small doses of up to 24 mg ZEA/day/animal administered through fodder during the same period did not produce any evident clinical effects over them, after the breeding period [42].

2.3.2. Transmission of zearalenone to milk of dairy cows

In general, it is considered that the transfer of ZEA and its metabolites in milk is very low [44].

Many researchers associate the reduced milk production, low fertility and hyperestrogenism at cows with the presence of ZEA in cereal or hay. Shreeve et al. ascertained that dairy cows fed with a ration containing 385–1982 μg ZEA/kg fodder, over 7 weeks, had a normal production of milk and there were no cases mycotoxin residues in milk, urine, serum or tissue [45]. In a 2004 study regarding the contamination with aflatoxin, ochratoxin and zearalenone, wheat and barley bran samples which were administered as a supplement to the food of dairy cows were analyzed. At the same time, determinations were done regarding the mycotoxin transfer in blood, milk and urine for the cows that consumed the contaminated feed. The results obtained showed the absence of aflatoxins AB_1, AB_2, AG_1 and AG_2, as well as the absence of ZEA (values under 5 ppb in fodder and under 1 ppb in serum, milk and urine) in the fodder samples analyzed (values under the detection limit of 0.1 ppb). In a significant proportion, of approximately 90%, ZEA is transformed in α-zearalenone whose toxicity is very high and, in a smaller proportion, in β-zearalenol. As in the case of OTA, protozoa are 9 times more active than bacteria in the degrading of ZEA [23]. The transformation at ruminal level of ZEA in zearalenol, together with the reduction in polarity, affects the absorption and excretion rate of the toxin thus, [23] in accordance with many other studies, reducing the elimination rate of ZEA and its metabolites in milk.

2.4. Trichothecene

Trichothecenes are a group of 43 mycotoxins (DON or vomitoxin, NIV, DAS, T-2 toxin etc.) with a similar chemical structure, developed by species of fungi from the following types: *Fusarium* (*F. graminearum*, *F. sporotrichioides*, *F. culmorum*, *F. poae*), *Myrothecium sp.*, *Phomopsis sp.*, *Stachybotrys sp.*, *Trichoderma sp.* and *Trichothecium sp.* [23].

From a chemical point of view, trichothecenes are derivative compounds of a tetracyclic sesquiterpene nucleus containing the epoxy- stable group in positions 12 and 13 and double C-C link in positions 9 and 10 [5].

Trichothecenes are metabolized *in vivo* in four ways: hydrolysis at the ester group level, hydroxylation, epoxy reduction and conjugation in the digestive tract, liver and other target organs of the animal organism [46]. The metabolization of trichothecenes is relatively simple, the halving time in the plasma varying between several minutes and several hours, depending on the mycotoxin. Within 24 hours of the oral administration, in the digestive tract of bovines were found both parental compounds and their metabolites, free and glucorono-conjugated [47].

In general, the DON, T-2 toxin and DAS mycotoxins do not accumulate in significant quantities in the organism, regardless of the administration method, since their metabolic compounds are eliminated from the organism within days. In certain situations though, there can be accumulations of the lipophilic trichothecenes, T-2 toxin and DAS, at the skin and fat tissue level. *In vitro* incubation in ruminal fluid of the DON mycotoxin, for 48 hours, determined its partial conversion into deepoxy-DON, metabolite non-toxic for ruminants.

Charmley et al. administered a ration of contaminated wheat and corn to 18 primiparous cows, formulated in order to induce a daily consumption of 0.59 mg, 42 mg and 104 mg DON. The authors saw that an increased concentration of DON in the ration did not affect fodder consumption or milk production. There were however modifications of the fat percentage in the milk and of the fat production, for the cows that received 42 mg of toxin daily. The authors did not observe the transfer of DON or deepoxy-DON in milk [48].

Ruminal microsymbionts can degrade DON resulting in the formation of 12,13-de-epoxide-oxinivalenol (DOM-1). Côté et al. ascertained, following the administration of a ration with 66 mg DON/kg fodder, the presence of the DOM-1 metabolite in amount of 30 μg/l in milk and the absence of the parental mycotoxin [49]. In a study on lactating sheep, Prelusky et al. administered 880 mg DON/kg fodder, for 3 days and highlighted the presence in the milk of 220 μg/l mycotoxin, of which the majority was DOM-1 [44].

In a study done in North Carolina, Whitlow et al. found a significant decrease in the production of milk at cows that consumed concentrated fodder contaminated with 0.8 mg DON/kg DM. Such a result can be explained through the synergic effect of mycotoxins associated with DON even though these were not identified. The presence of DON residue in the animal tissue was not identified in this study [50].

As is the case for other mycotoxins, studies regarding the adding of DON to fodder din not reveal the same toxicity compared to the food naturally contaminated with DON [51]. This is explicable due to the multiple interactions between mycotoxins in fodder, under natural conditions.

2.4.1. Micotoxicosis produced by trichothecenes

Trichothecenes produce a large variety of gastrointestinal disorders such as: vomiting, diarrhea, dermic inflammation or irritation, abortion, hemorrhages and immunosuppression.

Immunosuppression generated by trichothecenes is realized through a complex mechanism that makes the animals more sensitive to pathogen agents.

Deoxynivalenol (DON) or vomitoxin has a reduced impact on dairy cattle, clinical signals being associated between DON contamination of fodder and reduced performances in dairy herds, especially the reduction of milk production. A Canadian study on 18 first-lactation cows during mid-lactation, showed that the production of milk reduced with 13% or 1.4 kg when the cows consumed food contaminated with DON 2.6–6.5 ppm [48]. Meat cattle and sheep tolerated a diet with 21 ppm DON without visible effects on the health state or production [52].

Among the general effects of DON on the organism, we mention: inhibitor of protein synthesis, affliction of the gastrointestinal tract, immune system depression.

T-2 toxin is found in a relatively lower proportion in fodder compared to other trichothecenes, under 10% and, in general, data related to its effect on ruminant health are reduced. T-2 toxin reduces ingestion, lowers production and affects reproduction; depending on dosage and duration of toxin ingestion, it results in gastroenteritis, ulcers and death [53].

The hemorrhagic syndrome can be either absent although gastrointestinal injuries are produced as presented by Weaver et al. or present, combined with reduced ingestion, milk production and absence of estrus cycles in cows [43, 54].

2.5. Fusariotoxicosis

Fusariotoxicosis is a mycotoxicosis that manifests itself through a complex of clinical symptoms and injuries to the digestive and genital apparatuses, central and hematopoietic nervous system and of the blood, provoked by different toxins from some *Fusarium* species.

Etiologic agent. Fuzariotoxicosis is produced by the mycotoxins ZEA and DON developed by *F. graminearum* as well as other *Fusarium* species such as *F. nivale* and *F. tricinctum*.

Contamination sources are represented by cereals contaminated with *Fusarium*, most affected being corn grains. Development of *Fusarium* fungi is favored by high temperatures of 24–27°C, while the development of ZEA is favored by lower temperatures, of 12–14°C.

This ecologic characteristic explains the higher incidence of the mycotoxicosis in autumn or fall, when humidity is high and low and high temperatures alternate [29].

The toxic form begins at 5–6 hours from the consumption of contaminated fodder, with 1–4 days of evolution. It is manifested through salivation, chills, accelerated pulse and breathing, rumen hypotonia, teeth screeching. Clinically, we can also observe: loss of appetite, deviation, photophobia, arrhythmia, cutaneous hyposensitivity, exophthalmia, diarrhea, paresis, paralysis of the hindquarters.

The estrogenic form is rarely seen at taurines and is manifested through parturition and puerperal complications, metritis consequence of retained placenta, uterine involution, abortions,

heat cycle disruption, vaginal edema and prolapse, hypertrophy of mammary glands, etc. These clinical forms appear after consuming fodder contaminated with more than 24 ppm of ZEA [29].

Other species of *Fusarium* produce through the mycotoxins they develop various clinical manifestation in cattle. Thus, *F. nivale* develop the mycotoxins nivanelon, fuzarenon and BT butenolid, same as *F. tricinctum*, producing after being ingested by dairy cow's peripheral vasoconstriction and gangrene injuries of extremities due to ischemia; *F. tricinctum* develops the toxin T-2, F-2 and DAS. The toxin T-2 has inflammatory action over teguments and, in large quantities, can lead to skin necrosis. The consumption of fodder that contain the toxin T-2 leads to clinical manifestations such as loss of appetite, vomiting, severe dysentery, drop in coagulability of the blood and other signs of gastroenteritis [55].

Anatomopathological modifications in toxic form. At the necropsy exam hemorrhagic injuries can be seen, as well as catarrhal and sometimes hemorrhagic inflammation of the rennet, intestines and, as blood modification, leukocytosis with neutrophilia and eosinophilia.

Presumptive diagnosis is established on the basis of epidemiologic enquiry, clinical exam and anatomopathological exam, while the certainty diagnosis is established on the basis of paraclinical examination.

3. Combined mycotoxins

105 samples of fodder were analyzed, of which 75 samples of concentrated feed (cereal grains, wheat and maize bran, peas, sunflower and soybean meal) and 30 samples of fodder feeds from 5 family dairy farms in Southern Romania. The mycotoxicologic analysis was performed by the ELISA immunoassay test for AF, OTA, DON, ZEA and T-2.

In the 105 feed samples analyzed, in descending order, OTA was identified in a proportion of 63.80% (67 samples), T-2 in a proportion of 40.90% (43 samples), AF, ZEA and DON in a proportion of 39.0% (41 samples). By mycotoxin categories, in descending order, the maximum admissible limit in the 105 feed samples analyzed was exceeded in proportion of 40.95% for T-2 (43 samples), 33.30% for ZEA (35 samples) and 9.52% (2 samples) for OTA.

According to categories of feed, in descending order of the 30 analyzed fodder feed samples, the following were determined: 66.60% (20 samples) OTA, 36.60% (11 samples) ZEA, 33.30% (10 samples) DON, 26.6% (8 samples) T-2; in the concentrated feed analyzed, OTA was identified in proportion of 62.66% (47 samples), AF in proportion of 54.60% (41 samples), T-2 in proportion of 46.60% (35 samples), DON in proportion of 41.30% (31 samples) and ZEA in proportion of 40.00% (30 samples).

Of 105 analyzed feed samples, in decreasing order, 29.50% (31 samples) had two mycotoxins, 27.60% (29 samples) had three mycotoxins, 23.80% (25 samples) had one mycotoxin, 9.25% (10 samples) had four mycotoxins, 5.71% (6 samples) had no mycotoxins, and 2.85% (3 samples) had five mycotoxins.

Of the 25 samples with a mycotoxin, in decreasing order, the incidence was 9.52% (10 samples) for OTA, 4.76% (5 samples) for T-2, 3.80% (4 samples) for AF and ZEA and 1.90% (2 samples) for DON.

Of the 31 samples with two mycotoxins, in decreasing order, the incidence was: 6.66% (7 samples) for AF + OTA combination, 5.71% (6 samples) for OTA + T-2 combination, 4.76% (5 samples) for OTA + DON and AF + T-2 combination, 3.80% (4 samples) for OTA + ZEA combination and 0.95% (1 samples) for ZEA + DON; ZEA + T-2; DON + T-2 and AF + ZEA combination.

Of the 29 samples with three mycotoxins, in decreasing order, the incidence was: 6.66% (7 samples) for OTA + ZEA + DON combination, 3.80% (4 samples) for OTA + DON + T-2; AF + OTA + DON and OTA + ZEA + T-2; 2.85% (3 samples) for AF + ZEA + T-2 and 1.90% (2 samples) for AF + OTA + ZEA; AF + DON + T-2 and AF + OTA + T-2 combination.

Of the 10 samples with four mycotoxins, in decreasing order, the incidence was: 3.80% (4 samples) for AF + OTA + ZEA + DON + T-2 combination; 2.85% (3 samples) for AF + OTA + DON + T-2 combination; 1.90% (2 samples) for OTA + ZEA + DON + T-2 combination, and 0.95% (1 sample) for AF + ZEA + DON + T-2 combination.

Of the three samples with five mycotoxins, the incidence was 2.85% (3 samples) for AF + OTA + ZEA + DON + T-2 combination.

4. Prevention and treatment

Prevention of mycotoxin contamination of feed should start from the field, especially since the climatic condition indicate possible crops contamination with mycotoxigenic fungi: drought or heavy rain, aggression of harmful insects, other stress situations for plants (application of treatments, for example). A mycological and mycotoxicological analysis of feed at this stage would be indicated and would provide accurate and important information for subsequent prevention actions (during storage and feeding animals).

Also, the fungal growth and mycotoxin production conditions are not the same. Thus, *Aspergillus spp*. grows at a higher temperature and lower a_w compared to *Fusarium spp*.; the production of AFB_1 and AFB_2 by *Aspergillus flavus* in corn, for example, is favored by heat and drought stress associated with warmer climates and, furthermore, is enhanced by insect action both before and after harvesting. In fact, *Fusarium spp.* is one of the genres of fungi that develops both pre-harvest and post-harvest. On the other hand, fungal growth and association with Alimentary Toxic Aleukia has the best conditions at 25–30°C while the production of mycotoxins by *Fusarium* is not favored under this conditions; the opposite effect, strongly mycotoxigen, although fungi do not have a high growth rate, is observed at near-freezing temperatures [56]. Similar situations can be also observed when applying fungicides that reduce the growth of fungi but not the mycotoxins [57].

During the storage period, maintaining optimum conditions: less than 14% humidity in the feed, creating optimal conditions for silage - quickly reducing pH and elimination of oxygen, microbial or enzymatic silage additives, may partially inhibit the development of mycotoxigenic fungi or reduce their ability to produce mycotoxins. However, if contaminated feed is still in the animal feed, a dilution of it with healthy fodder is recommended. A number of mycotoxin-adsorbing agents can also be used as food supplements: sodium and calcium aluminosilicates, bentonites, montmorillonites, zeolite, some organic polymers (polysaccharides, glucomannans, peptidoglicans, etc.), activated carbons, yeast cell walls, micronized fibers, bacteria. As

mycotoxin-biotransforming agents: gram-positive anaerobic and aerobic bacteria, gram-negative aerobic bacteria, fungi, yeast, enzymes (e.g. *Flavobacterium aurantiacum* for aflatoxins, *Eubacterium* BBSH 797 and LS100 for trichothecene, and for OTA and ZEA, *Trichosporum mycotoxinivorans*; protease A, pancreatin etc) [58]. Galvano et al. have shown that an increase in the level of some nutritional parameters in food – protein, energy and antioxidants, mineral and vitamins can be beneficial to animal health by mitigating the harmful effects of mycotoxins [59].

5. Conclusions

The increase and diversification in the production of fodder, particularly cereal, through new technologies has direct consequences on the change in their chemical composition and, implicitly, over the growth and development of fungi before the harvest, during the transportation or during the storage of fodder. Moldy feed has reduced palatability, which certainly determines reduced ingestion and implicitly a drop in milk production and, afterwards, in corporeal weight. There are unanimously accepted losses of 5–10% of the performances of milk cows under the condition that they ingested fungi contaminated fodder, irrespective of the latters' contamination with mycotoxins. Mold growth and mycotoxin production are strongly linked with the action of certain predisposing factors such as extreme weather conditions (draught followed by rain, for example), the favoring action of harmful insects and can be produced in the field, during transport, processing or even while the fodder is administrated to the animal. The risk of affecting the health of ruminants due to the action of mycotoxins is much larger compared to that of the action of fungi. Among the mycotoxins that can affect the health of milk cows and, implicitly the reduction in production, aflatoxins are certainly the most aggressive. The risk is proportionally higher with their metabolites, as aggressive as them, reaching the milk production and affecting human health.

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Chapter 6

The Use of Serum Proteins in the Laboratory Diagnosis of Health Disorders in Ruminants

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.72154

Abstract

Although hundreds of proteins exist in blood serum, little is known about the precise composition and entire set of serum proteins in different ruminant species. Under physiological conditions, the production of serum proteins is closely regulated, but alterations in the serum protein pattern may occur in a wide range of diseases and health disorders. During the last several years, substantial progress was seen in the application of serum protein analyses for diagnostic purposes. The serum protein profile is mostly evaluated by serum protein electrophoresis, which allows the identification of protein fractions, each being composed of several individual proteins with similar electrophoretic mobility. Many disease processes can cause changes in the concentrations of serum proteins. Therefore, the determination of their concentrations and the evaluation of changes in their concentrations during the disease process may provide important diagnostic information for assessing the health state. Despite this usefulness, the evaluation of serum protein pattern is still relatively a less frequently used laboratory diagnostic technique in ruminant medicine. Thus, the usefulness of serum proteins in the diagnosis of health disorders and the possible clinical application of the results of the electrophoretic separation of serum proteins in ruminants will be reviewed in this chapter.

Keywords: acute-phase proteins, blood proteins, dysproteinemia, electrophoresis, laboratory diagnostics, ruminants

1. Introduction: serum proteins

Proteins are the main and most abundant constituents of the blood serum or plasma, having many essential physiological functions. The most of proteins present in the blood are biochemically not pure; usually, they are a mixture of simple proteins combined with other

substances: glycoproteins, lipoproteins, and other conjugated proteins [1]. Proteins have a specific intramolecular structure and amphoteric nature, containing the balanced portions of hydrophilic and hydrophobic groups [2]. They are macromolecules built from one or more unbranched chains of amino acids linked by peptide bonds. The chemical properties of the amino acids determine the biological activity of the protein [3].

Proteins play a central role in biological processes; some of them are involved in structural support of connective tissues, while others play important roles in biochemical reactions. Proteins also serve as buffers, helping in maintaining the acid-base balance and colloid osmotic structure. Some of them act as carriers of lipids, hormones, vitamins, and minerals in the circulatory system, and are involved in the regulation of cellular activity and immune system [4]. Other blood proteins play important roles as enzymes, complement components, or protease inhibitors. Certain blood proteins are essential for hemostasis and have important functions in platelet adhesion and aggregation, as well as coagulation [5].

Hepatocytes play the major role in the synthesis and secretion of blood proteins [6]. The major exceptions are the immunoglobulins that are produced by the immune system consisting of the reticuloendothelial tissues, lymphoid cells, activated B cells, and plasma cells in response to exposure to antigens [7, 8]. Further studies showed that nonhepatic tissues, including the intestine, lung, adipose tissue, and mammary gland, also have the capability to synthesize some serum proteins for specific functions [9, 10].

The protein constituents of the blood serum are qualitatively different from that of plasma, in which fibrinogen has been removed by conversion into a fibrin clot together with some other coagulation factors [11, 12]. Although serum and plasma are considered suitable samples for many chemistry tests, including serum total proteins, differences in the results obtained between these 2 sample types have been reported by some authors [13, 14]. The principal advantage of plasma over serum is the smaller amount of blood specimen that can be collected from some small animal species. In these species, heparinized plasma is the preferred sample for clinical chemistry, including protein analyses [15]. However, according to some authors, plasma and serum are not equally suitable samples for protein analyses due to noticeable differences in the electrophoretic pattern of proteins in serum and plasma. It should be taken into consideration that fibrinogen, which migrates at the end of the β region on the electrophoretogram, may influence the correct separation and identification of protein fractions [16] (**Figure 1**). Errico et al. [17] concluded also that electrophoretic analysis of proteins in plasma may provide inaccurate results unless plasma is defibrinated, caused predominantly by the possible overestimation of the β-globulin fraction.

The concentrations of proteins in serum are tightly controlled to balance their physiological functions in areas of immunity, coagulation, small molecule transport, and inflammation. Any dysfunction and out-of-balance in their concentrations can cause or result from disease processes [18]. Blood serum contains many different proteins. Some of them are present in the blood serum in concentrations higher than mg/ml, including albumin, immunoglobulins, haptoglobin, transferrin, and lipoproteins [19]. In addition to these major constituents, blood serum also contains many other proteins that are secreted by cells, and tissues in very low concentrations (measured in ng/ml or pg/ml) and in veterinary clinical biochemistry are relatively underutilized [20, 21].

http://dx.doi.org/10.5772/intechopen.72154

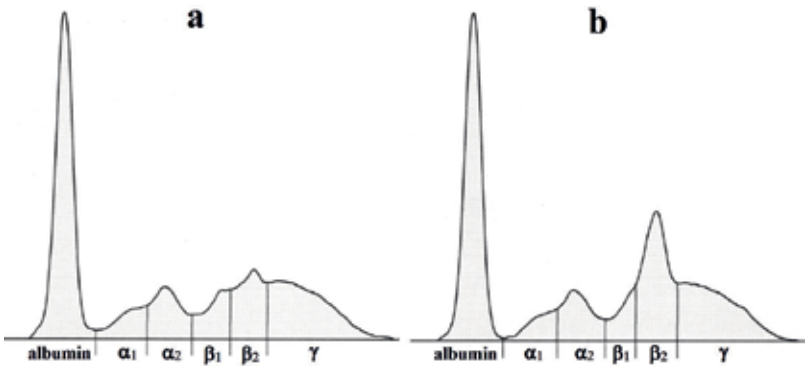

Figure 1. Normal serum (a) and plasma (b) protein electrophoretogram in a clinically healthy cattle.

2. Analyses of serum proteins

Blood proteins are an important indicator of health state and their evaluation represents a basis in general biochemistry [22]. The first step in the analysis of protein pattern is the quantification of total serum or plasma protein concentrations. Several methods have been developed for their determination, which are based on different analytical methods [23]. Several techniques, including chemical and physical methodologies, may be applied to analyze the concentrations of total proteins in blood and other biological fluids. Chemical methods belong to the more commonly used procedures in clinical biochemistry, since they may be to adapt to automated analysers [7].

2.1. Chemical methods

In biochemical laboratories, the most widely used analytical technique to assess the concentrations of total proteins is the biuret method. This method is based on colorimetric principle, in which the copper ions from the biuret reagent react with the amide groups from the proteins at strong alkaline pH, creating a violet color [24, 25]. However, this method is not sensitive enough to measure lower protein concentrations found, for example, in cerebrospinal fluid [26]. Despite of this disadvantage, the biuret assay is still frequently used because of simple analytical procedure, easy preparation of reagents, and when compared with other copper-based assays, this method is less susceptible to chemical interference [27]. Many of the total protein assay kits developed for the automated use in wet biochemical analyzers, as well as dry chemistry analyzers, are based on this principle. This technique is very cheap and this favored its wide application in veterinary medicine.

The biuret method was modified by using the Folin phenol reagent (Folin-Ciocalteu), which is more sensitive and thus more appropriate to measure low concentrations of proteins [28]. In this method, the phenolic groups of tyrosine and tryptophan in proteins react with the Folin-Ciocalteu reagent producing a blue-purple colored complex [29]. The disadvantages of the Lowry method are the sensitivity to the amino acid composition of the protein and the interference with a range of substances, including buffers, drugs, and nucleic acids [30].

Another method for the determination of protein concentrations is the Bradford assay, which is based on the binding of the Coomassie brilliant blue dye to the proteins in an acidic solution to form a complex with increased molar absorbance [31]. This assay is rapid, practical, and suitable for simple quantification of proteins in cell lysates, cellular fractions, and recombinant protein samples [32]. It may be performed also in microtiter plates using micro volumes, but its application area is mainly restricted to research laboratories [33]. Unfortunately, the Bradford assay is linear over a short range (to 2000 μg/ml) and shows a curvature over this range of protein concentration, which necessitates the dilution of samples before further analysis [34, 35].

2.2. Physical methods

The concentrations of serum or plasma proteins may be measured also by physical methods. Refractometers are used by many veterinary practitioners, because of their ability to measure the protein concentrations in various biological fluids rapidly. Generally, the refractometric technique is based on the determination of the extent, how light is refracted when it passes from one medium to another of different densities (usually from air into the sample) [36]. The angle of refraction is proportional to the concentration of solute in solution. Seeing that proteins are the most important solute dissolved in serum, the refractive index indicates the concentration of proteins in the sample [37]. A good correlation between refractometry and the biuret method was found in human serum samples [38], but the results for veterinary samples are less consistent. Indeed, whether some authors have reported a good correlation of results for domestic mammals (biuret methods vs. refractometry), others showed either higher or lower values for refractometry compared to the biuret method [39, 40]. The differences between the methods were of 6 g/l and 2 g/l in dogs and cats, respectively [36]. However, the most marked differences between the biuret and refractometric methods were observed in avian samples due to the interference by high concentrations of other light-refractive non-protein components of the blood, such as glucose, cholesterol, or lipids [37, 41]. These variations might be caused by differences in the design of various refractometers assigned by the manufacturers, variation in the biuret reagent mixture, as well as assay [42]. Vandeputte et al. [43] evaluated four different refractometers for measuring serum total protein concentrations in beef calves in comparison with the results obtained by the biuret method. In this study, the refractometric measurements were highly correlated with those obtained by the biuret method indicating similar accuracy for measuring serum total protein values. Calloway et al. [44] and Wallace et al. [45] identified a similar ability to detect failure of passive transfer in calves with refractometers. As the index of refraction is influenced by the temperature of the solute, Automatic Temperature Compensation (ATC) refractometers were commercialized to avoid the impact of potential temperature variations on the results [43]. Recently, digital refractometers have been introduced also into the veterinary medicine, where they demonstrated excellent precision with good sensitivity and specificity [43, 45]. However, according to Hunsaker et al. [46], they did not introduce benefits in accuracy over manual refractometry in regards to potential interference due to non-protein solutes.

http://dx.doi.org/10.5772/intechopen.72154

3. Evaluation of protein fractions and individual proteins

The identification and quantification of individual serum proteins or groups of proteins are possible only if they are separated. In the protein analyses, the most important method available to measure independent proteins or protein groups is the fractionation technique. Blood serum consists of a large number of proteins; thus, the whole protein complex is not possible to analyze in a simple step by currently available separation technologies [47].

The two major types of proteins in the blood are albumin and globulins. Currently, the bromocresol green (BCG) and bromocresol purple (BCP) methods are the basis for the determination of serum albumin [48]. The BCG method is a dye-binding technique characterized by an ionic interaction between positively charged albumin and negatively charged dye molecules at acidic pH [49]. The bromocresol green binds quantitatively with albumin forming an intense blue-green complex, and the intensity of the color produced is directly proportional to the albumin concentration in the sample [50]. This method is easy to perform, rapid, and cheap, but less sensitive and selective compared to immunoassays [51]. Factors such as optimal pH, ionic strength of buffer, sample preparation, dilution rate, incubation time, and interfering proteins may affect the accuracy of this technique [52, 53]. The reaction between serum and BCG is not specific for albumin; therefore; the BCG method often overestimates the concentrations of serum albumin, but its specificity can be improved by minimizing the contact time with the serum sample [54]. The BCG method is often used to determine the serum albumin concentrations also in animals, including ruminant species [55]. However, albumin methodologies in chemistry analyzers are optimized and designed to measure human albumin. Furthermore, bromocresol green can bind animal globulins with extended reaction times [56, 57]. Therefore, protein electrophoresis may be a better method to provide more accurate albumin quantification [58].

Bromocresol purple is an another related dye that may be used for the determination of albumin concentrations, giving more accurate results and thus has better diagnostic utility [54, 59]. Bromocresol purple is an albumin selective dye, which minimizes globulin interference that occurs with bromocresol green by long incubation (more than 30 seconds) [60, 61]. Good correlation was observed between the serum albumin values obtained by the BCP method and immunoassay [61, 62]. Discrepancies may be observed between the serum and plasma albumin values determined by the BCP method. Plasma albumin concentrations may be falsely increased by turbidity due to the precipitation of fibrinogen when plasma is diluted into the BCP reagent [63, 64].

A number of methods have been developed to measure the concentration of globulins. One type of these techniques is based on the precipitation of globulins using solutions of metal salts, e.g., sodium sulfite or zinc sulfate [65, 66]. The addition of salts causes turbidity, which may be visually evaluated or measured by spectrophotometer as units of turbidity. This method may be used as a field test for the evaluation of suckling efficiency or failure of passive transfer of maternal immunity via colostrum in calves and foals [67–69]. However, protein electrophoresis is recommended to accurately determine globulin distribution, allowing to efficiently and precisely detect, as well as quantify several globulin fractions (α-, β-, and γ-globulins) [70].

3.1. Protein fractionation

Electrophoresis is the current standard and most widely used fractionation technique of serum proteins in clinical biochemistry and molecular biology [71]. Several fractionation techniques have been developed to separate and consequently quantify the proteins in serum [72]. The most of them depend on the initial determination of total serum proteins, and then the concentrations of the main fractions can be calculated from the total protein values. Electrophoresis is based on the movement of charged particles through a buffered medium when subjected to an electrical field [73]. Serum proteins have a negative charge, so in the electrophoretic chamber, they migrate toward the positive pole in an electrical field and are separated from each other in different bands according to their sizes [74]. The speed of their movement depends on the characteristics of the protein undergoing separation, including its electrical charge, size and shape, as well as on the strength of the electrical field, type of medium used for the separation, and temperature [75]. After separation, the protein fractions are fixed in an acid solution to denature the proteins and immobilize them on the support medium [3]. The proteins are then stained and quantified by density measuring, providing also graphical data for computer analysis according to the used electrophoretic system [76].

The separation of proteins in an electric field was introduced by Tiselius in the 1930s [77]. The application of serum protein electrophoresis in clinical biochemistry laboratories started in 1950s using paper strips [78], were replaced a few years later by microporous acetate membranes [79]. In the 1970s, agarose gel as a support medium was introduced in the electrophoretic separation of proteins [80], and became a most commonly used supporting substance in veterinary medicine. There are great differences between the electrophoretic methods, which are usually caused by the material of support medium used for the fractionation of proteins [81]. Luraschi et al. [82] stated that the electrophoretic patterns of proteins and the numbers of identified peaks are dependent on the used support material: cellulose acetate vs. agarose gel electrophoresis. The standard agarose gel electrophoresis is a labor-intensive method, but the introduction of prepackaged gels and the development of new equipments allowed the automatization of this procedure [81, 83]. However, the correction of electrophoretograms by visual inspection of an experienced interpreter is very important. Furthermore, agarose gel electrophoresis has several advantages compared to cellulose acetate. Indeed, agarose gel as a support medium provides better resolution, higher reproducibility of results, and greater clarity of the electrophoretic bands [84].

In past few years, capillary zone electrophoresis (CZE) is being used also in veterinary laboratories [85]. In CZE, the separation of protein fractions occurs in a free liquid medium created by the low viscosity buffer, in which the application of high voltage generates an electroosmotic flow causing rapid movement of proteins toward the cathode [86]. This allows better separation of proteins with similar physicochemical characteristics, thus generating multiple subpeaks or narrower peaks [87]. The higher resolution of CZE can often result in abnormal electrophoretic profiles caused by the aforementioned multiple subpeaks of unknown significance, which is the disadvantage of this method. Recently, laser densitometry was introduced for a precise tracing of the electrophoretic separation [88].

http://dx.doi.org/10.5772/intechopen.72154

Electrophoresis in ruminant species is normally used in serum, but plasma or other body fluids (urine, cerebrospinal fluid) may also be processed. Serum is the best material for protein electrophoresis, as it does not contain fibrinogen. Electrophoretic technique may be used also for the analysis of urinary proteins, which is a fundamental step in the early diagnosis and subsequent monitoring of renal diseases [89]. It was found that polyacrylamide gel electrophoresis may localize the origin of urinary proteins based on their molecular weight, providing a diagnostic sensitivity comparable to results obtained by kidney biopsy [90]. It is considered a very sensitive method to discriminate between glomerular, tubular, or mixed proteinuria [91]. Agarose gel electrophoresis may be applicable also to separate the main protein fractions in cerebrospinal fluid (CSF) samples. Evaluation of proteins in CSF may provide important information about the production of immunoglobulins within the central nervous system, as well as possible disturbances in the blood-brain barrier [92]. Cerebrospinal fluid contains only a small amount of proteins when compared with serum. Therefore, the proteins in CSF should be concentrated to increase the sensitivity of CSF protein detection by electrophoresis [93].

3.2. Analysis of individual serum proteins

Recently, several techniques, including high resolution electrophoresis, two-dimensional electrophoresis, and proteomic assays were developed for the separation of proteins. These methods allow simultaneous identification of many individual proteins and localize specific proteins within several subfractions. On the other hand, the agarose gel electrophoresis is able to separate serum proteins only into five or six fractions [7]. Changes in proteins with very low concentrations may not be detected by electrophoresis [22]. Immunoassays are another type of methods that may be used for the determination of specific serum proteins. These procedures require a specific antibody against the analyzed serum protein. In biomedical research, enzyme immunoassay (EIA) and enzyme-linked immunosorbent assay (ELISA) belong to the most common analytical methods, which may be used for the identification and quantification of specific proteins, antigens or antibodies [94]. ELISA is based on the concept of an antigen/protein binding to its specific antibody, which allows to detect very small concentrations of antigen/protein [95]. Various types of ELISAs have been developed, while the basic step is the direct or indirect detection of antigen by adhering or immobilizing the antigen or antigen-specific capture antibody onto the well surface [96]. Direct ELISA is considered to be the simplest format of immunoenzymatic assays that determine an antigen immobilized to the plate using an antibody directly conjugated to an enzyme [97]. The indirect ELISA technique requires a secondary antibody to detect the presence of antigen, which is "sandwiched" between the capture antibody coated on the plate and an enzyme-labeled conjugate. Furthermore, the determination of some serum proteins is possible based on their biological activities. For example, the high affinity of haptoglobin for hemoglobin may be used to assess its concentrations. Subsequently, the peroxidase activity of the bound hemoglobin is maintained at low pH [98], the intensity of which is directly proportional to the concentration of Hp in the sample. This colorimetric reaction is not species specific and may be used in several animal species, including ruminants. On the other hand, ceruloplasmin has endogenous oxidase activities, which can be applied to measure its concentrations [99]. However, for the quantitative determination of the most of serum proteins in animals, species-specific assays should still be developed.

4. Physiologic serum protein pattern in large and small ruminants

Following electrophoresis, serum proteins can be separated into four basic fractions including albumin, alpha(α)-, beta(β)-, and gamma(γ)-globulins [100]. Each band consisted of many individual proteins having various metabolic activities. The electrophoretic pattern of serum proteins and its interpretation are related to differences observed among various animal species, as well as among different groups of animals. Great species-specific variations in the type and size of serum protein fractions were observed by many researchers [101, 102].The number, shape, and size of fractions and subfractions change a lot with the animal species and breed [103]: the most important differences are inside β-globulins and even γ-globulins. Differences in the electrophoretic mobility of serum proteins have been observed also between ruminant species (**Figure 2**).

Nagy et al. [104], by using agarose gel electrophoresis, described six fractions in bovine serum comprising albumin, α_1- and α_2-, β_1- and β_2-, and γ-globulins. Whereas, Alberghina et al. [105] and Piccione et al. [106] separated the bovine serum proteins into five fractions, comprising albumin, α_1-, α_2-, β-, and γ-globulins. The number of protein fractions in sheep and goat serum varied between various authors. Nagy et al. [104] and Esmaeilnejad et al. [74] in sheep serum recorded albumin, α_1-, α_2-, β-, γ_1-, and γ_2-globulins, while the goat serum proteins showed albumin, α_1-, α_2-, β-, and γ-globulin fractions [104, 107]. In contrast, Cyrillo et al. [108], Fernandez et al. [109], and Alberghina et al. [102] determined only one α-globulin and two β-globulin fractions in goat serum.

4.1. Prealbumin (transthyretin)

Prealbumin (transthyretin, TTR) is the most rapidly migrating protein fraction in serum visible as a band anodic to the main albumin fraction on the electrophoretic gels [79]. According to Hamilton and Benson [110], this property is attributed to human prealbumin, not to bovine. Kaneko [22] stated also that prealbumin is not always visualized in electrophoretograms and may not exist in all animal species, including ruminant species. Therefore, in these animals, species-specific ELISA assays should be used for the detection and quantification of transthyretin.

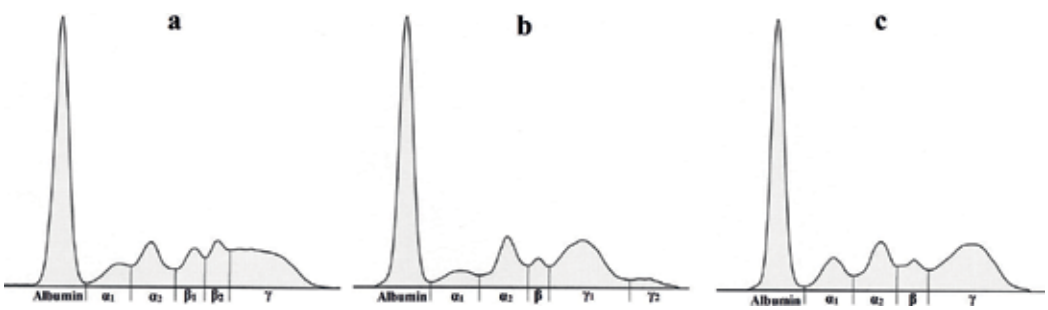

Figure 2. Representative agar gel electrophoretogram in a cow (a), sheep (b), and goat (c) [104].

http://dx.doi.org/10.5772/intechopen.72154

Transthyretin is a small globular non-glycosylated tryptophan-rich protein of a homotetrameric structure, composed of four identical subunits with two thyroxine binding sites per tetramer [111]. The main physiological functions of TTR include the carriage of thyroid hormones [112]. Another important function of TTR is the transport of retinol (vitamin A) through its association with retinol-binding protein (RBP) from its main storage site in the liver to target cells [113]. From this reason, in the 1980s, the name prealbumin was changed to transthyretin (TTR) describing its ability to bind both thyroid hormones, and retinol binding protein (RBP) [114]. Furthermore, transthyretin acts as a negative acute-phase reactant, serum concentrations of which fall due to decreased synthesis in inflammation, trauma, tissue injury, or stress [115]. It is synthesized mainly by hepatic parenchymal cells and in the choroid plexus of the brain, which has the highest concentration of TTR in the body [116, 117]. In cerebrospinal fluid, it is the second most abundant protein [118]. The major sites of transthyretin degradation are the liver, muscles, and skin [119]. It has a half-life in blood serum of approximately 2 days, which is much shorter than that of albumin [120]. Transthyretin is, therefore, more sensitive to changes in protein-energy status, and thus may be used as an indicator of malnutrition [121].

The concentrations of TTR in blood serum may be affected by many factors, including age, gender, as well as blood-drawing methods. A marked increase of TTR values from 72.9 to 251.4 mg/l was observed by Tóthová et al. [122] in calves 1 day after colostrum intake with a consecutive gradual decrease till the end of the third month of life. Rona [123] described that bovine colostrum contains, among other bioactive molecules, a small amount of prealbumin (transthyretin). Thus, the increase of serum TTR concentrations observed in calves after colostrum intake may reflect the adequate nutrition, as well as its hepatic synthesis due to adequate protein and energy intake [112]. The effect of hormonal changes during pregnancy on the concentrations of TTR in animals has not been reported. Our findings suggest no significant changes in TTR concentrations during the last week of pregnancy and early stages of lactation in dairy cows (unpublished data). The usefulness of prealbumin in the clinical and laboratory diagnosis of diseases was evaluated in dogs with nonthyroidal illness (including neoplasia, allergy, cardiac disease, gastrointestinal disease, parasitism, and hepatic disease) and in pigs with *Streptococcus suis* type 2 infection, showing its lower concentrations compared with healthy ones [124, 125]. In cattle, there are very little published reports about the use of prealbumin in the diagnosis of diseases. Our preliminary results suggest lower concentrations of TTR in diarrheic calves at the age of 1 month compared with healthy animals at the same age. Similarly, *Mycobacterium avium paratuberculosis* seropositive cows showed lower TTR values than those obtained in healthy cattle (unpublished data).

4.2. Albumin

Albumin is the most abundant protein found in blood plasma or serum, and essential part of the biochemistry profile. It is a homogenous protein fraction and is visible as a discrete zone on the electrophoretogram. In animals, 35–50% of the total serum protein concentration is made up from albumin [22]. The shape and size of albumin fraction are very similar in all ruminant species, which are related to its high serum concentration, homogenous electric charge, and high staining affinity. However, there are great differences in its relative

concentrations between different animal species [126]. Albumin can be seen on the left side of the electrophoretogram closest to the anode, where forms a large peak [76].

Albumin is small size protein with a molecular weight of 69 kDa. The main functions of albumin are the maintenance of homeostasis and transportation of substances, and it also acts as a free-radical scavenger [127]. It is responsible for about 75% of the osmotic pressure of plasma and is a major source of amino acids that can be utilized by the animal's body when necessary [128]. It also serves as a carrier protein for many insoluble organic substances (e.g., unconjugated bilirubin). Serum albumin is the major negative acute-phase protein. The synthesis of positive acute-phase proteins is markedly increased during the acute inflammatory processes. These reactions require a great amount of amino acids. Thus, albumin synthesis is downregulated and amino acids are used mainly for the synthesis of the positive acute-phase proteins [129]. Catabolism of albumin occurs in various tissues, where it enters cells by pinocytosis and is then degraded by proteases [130]. The major sites of these catabolic processes are muscle, liver, and kidney. There are major species-specific differences in the turnover of albumin, reflecting the body size. The half-time for clearance of albumin varies from 1.9 days in the mouse to 14–16 days in ruminants, and because of this, it may serve as a marker of chronic nutritional status [131]. Furthermore, may studies have established albumin as an indicator of morbidity and mortality [132].

4.3. Globulins

The globulin fractions may be found on the right side of the electrophoretogram. These peaks include a very heterogenous group of proteins, and depending on the species, there may normally be one or two α, one or two β, and one or two γ fractions [22].

4.3.1. The α-globulins

The α fraction is the most rapidly migrating protein of all the globulins, and in most species, it migrates as α_1 (fast) and an α_2 (slow) fraction. Many diagnostically important acute-phase proteins migrate in this fraction. Alpha$_1$-antitrypsin, α_1-acid glycoprotein, α_1-antichymotrypsin, α_1-fetoprotein, serum amyloid A, and α_1-lipoprotein have been identified in the α_1-globulin fraction, while haptoglobin, α_2-microglobulin, α_2-macroglobulin, ceruloplasmin, α_2-antiplasmin and α_2-lipoprotein in the α_2-globulin fraction [100, 133]. Acute-phase proteins are a large and varied group of serum proteins, with numerous differences in their concentrations between different animal species [134]. Their concentrations change in response to any alterations in homeostasis or tissue injury. They have specific functions in the regulation of inflammatory processes, predominantly at the site of inflammatory lesions, but they may act also systemically [115]. In general, the main function of the acute-phase proteins is to defend the host against pathological damage, remove the causative agents of disturbances, assist in the restoration of the homeostasis and in the regulation of different stages of inflammation [135, 136]. Moreover, some proteins from these fractions may act as inhibitors of enzymes, as digest proteins, as compounds of the blood coagulation system or as carrier of copper [71].

http://dx.doi.org/10.5772/intechopen.72154

4.3.1.1. Alpha-1 antitrypsin

Alpha-1 antitrypsin (AAT) is the major inhibitor of serine proteases (serpin) such as neutrophil elastase and proteinase-3 in the blood [137]. It is also an acute-phase protein. In some acute-phase inflammatory reactions, the concentrations of AAT may increase in order to limit the damage caused by activated neutrophil granulocytes and their enzyme elastase, thus limiting the tissue injury caused by proteases at the site of inflammation [138]. The clinical importance of AAT is underlined in patient with AAT deficiency, a hereditary disorder that can lead to severe tissue breakdown during inflammation [139]. Consequently, pulmonary emphysema, chronic obstructive lung disease, liver diseases, as well as liver cirrhosis may occur in these patients. In addition, liver cells produce an abnormal protein, which may accumulate in the body, leading to inflammation and/or cirrhosis of the liver [140]. From animal species, Sevelius et al. [141] measured the concentrations of alpha-1 antitrypsin in dogs and evaluated whether AAT aggregates could initiate liver disease. In cattle, little is known about the diagnostic utility of alpha-1 antitrypsin.

4.3.1.2. Alpha-1 acid glycoprotein

Alpha-1 acid glycoprotein (AGP) or orosomucoid is a highly glycosylated protein of which about 45% is carbohydrate and the composition of the glycan residues is known to alter during an acute-phase response [142]. AGP is considered as a natural anti-inflammatory and immunomodulatory agent. It has also been suggested that AGP is required to maintain capillary permeability [142]. Furthermore, AGP is one of the most important drug-binding proteins in plasma that can have important pharmacokinetic implications [143]. It has a moderate acute-phase response in most animal species and is more likely associated with chronic conditions. The serum concentration of AGP may be a valuable differential diagnostic analyte in the identification of feline infectious peritonitis [144].

In ruminant species, the concentrations of AGP were evaluated by Tóthová et al. [145] in calves during the first month of life. In this study, the AGP values were roughly uniform shortly after birth with an increase of values from the day 2 of life till the end of the first month of age, probably related to the normal process of growth, exposure of animals to changing environmental conditions, and nutritional factors. Similar findings were demonstrated by Rocha et al. [146]. On the other hand, the highest concentrations of AGP in the plasma were found by Itoh et al. [147] in calves immediately after birth (1368 μg/ml), gradually decreasing to 249 ± 100 μg/ml during the first 3 days of life, which are comparable to physiological values in adult bovine. Similarly, high plasma concentrations of AGP were observed by Orro et al. [148] in calves after birth, which was followed by a decrease during the first 3 weeks of life to adult values. The very high concentrations of AGP in the fetal stages may be related to synthesis of AGP in the embryonic liver [147]. These studies indicate that the production of AGP in the neonatal period is fetally regulated and its high serum concentrations after birth are not necessarily a sign of the activation of the acute-phase response by external stimuli.

4.3.1.3. Alpha$_1$-fetoprotein

Alpha$_1$-fetoprotein (AFP) is the predominant serum protein in the bovine fetus, which is mainly produced by the yolk sac and at the later period by the fetal liver [149]. Because of its biochemical similarity to albumin, AFP could be a carrier protein, or even take part in the metabolism of bilirubin [150, 151].

Smith et al. [152] have demonstrated that the concentrations of AFP in fetal bovine plasma reach the highest values in the 3–4th of fetal period, which is followed by significant decrease until birth. A decrease of the values of AFP in the first hours of life was obtained by Bader et al. [149] that may represent the physiological effect on the fetal tissues changing from an intrauterine to an extrauterine environment, with decrease in the production of fetal proteins in the liver. Lee et al. [153] concluded that following the initial decrease of the values of AFP within hours after birth, the concentrations of AFP tend to stabilize during the rest of the first week, and then decrease rapidly. On the other hand, Tóthová et al. [122] found a marked increase of AFP concentrations 1 day after colostrum intake, with following gradual decrease of values up to day 30 of life. The relatively higher values of AFP after birth may be associated with its synthesis (not ceasing entirely at birth) by fetal hepatocytes that continue during the early postnatal period [154]. Furthermore, colostrum contains many non-nutrient substances and immune factors, including alpha$_1$-fetoprotein, which may be responsible for the increased concentrations of AFP in calves 1 day after colostrum intake [155].

Alpha$_1$-fetoprotein may be used as a tumor marker to help detect and diagnose cancers of the liver, testicles, and ovaries. Sturgeon et al. [156] reported increased concentrations of AFP in approximately 70% of patients with hepatocellular carcinoma. Increased AFP concentrations were found also in 50–70% of patients with nonseminomatous testicular tumors. In veterinary medicine, the possible use of AFP as disease marker was not yet evaluated.

4.3.1.4. Serum amyloid A

Serum amyloid A (SAA) is a small hydrophobic protein that belongs to the family of apolipoproteins associated with high density lipoprotein [157]. Different isoforms of SAA are expressed constitutively at different levels in response to inflammatory stimuli [158]. During inflammation, SAA1 and SAA2 are expressed principally in the liver, whereas SAA3 is induced in many distinct tissues, including the mammary gland [159]. The fourth isoform, SAA4, does not respond to external stimuli [160]. The main functions of SAA are the reverse transport of cholesterol from tissue to hepatocytes, opsonization, inhibition of phagocyte oxidative burst, and platelet activation [136]. The M-SAA3 isoform found in colostrum stimulates the production of mucin from intestinal cells and thus helps to prevent bacterial colonization [161].

In ruminants, SAA belongs to major acute-phase proteins which increases more in acute rather than in chronic conditions [162]. Intense changes in the concentrations of SAA were reported in dairy cows with various inflammatory diseases, including cows with endometritis, mastitis, as well as in lame cows [163–165]. It was raised also in cattle experimentally infected with *Mannheimia haemolytica* and bovine respiratory syncytial virus, or with bovine viral diarrhea virus [166, 167].

Eckersall et al. [168] found significantly elevated SAA concentrations in sheep with experimental caseous lymphadenitis induced by *Corynebacterium pseudotuberculosis*. Chalmeh et al. [169] observed in sheep, a rapid increase of SAA values during experimentally induced endotoxaemia by lipopolysaccharide from *Escherichia coli*. Another study conducted by El-Deeb [170] showed an increase in the concentrations of SAA in ewes with pregnancy toxemia. Marked increase of SAA concentrations was recorded also in sheep following experimental infestation with *Psoroptes ovis* [171]. After treatment, the SAA values decreased rapidly within 3 days and returned to the pre-infestation values for 10–14 days. The alterations in the acute-phase protein production during experimental caprine coccidiosis were evaluated by Hashemnia et al. [172]. They found markedly higher concentrations of SAA at day 7 after inoculation. Furthermore, the magnitude and duration of the acute-phase responses are correlated well with the severity of the clinical signs and diarrhea in goat kids.

4.3.1.5. Haptoglobin

Haptoglobin (Hp) is a glycoprotein that consists of two α and two β chains, connected by disulfide bridges [173]. In the circulation, Hp is highly polymerized, having a molecular weight of approximately 1000–2000 kDa, and exists also as a polymer associated with albumin [174]. The primary function of Hp is to bind free hemoglobin released from erythrocytes and thereby inhibits its oxidative activity [175]. The Hp-hemoglobin binding also reduces the availability of the heme residue from bacterial growth [176].

Many studies have indicated the significance of Hp as a clinically useful parameter for measuring the occurrence and severity of inflammatory responses in cattle with various diseases, including mastitis, enteritis, peritonitis, pneumonia, as well as endocarditis [136, 177]. Higher concentrations of Hp were found also by Sheldon et al. [178] in cows with uterine bacterial contamination. In addition, Hp was detected in ewes as prognostic indicator of ovine dystocia [179]. Gonzalez et al. [180] studied the possible use of acute-phase proteins as markers of subacute ruminal acidosis in goats. They found a moderate increase of Hp concentrations during the induction period, while SAA did not change. In a further study, Gonzalez et al. [181] determined the effect of fasting-induced pregnancy toxemia on the concentrations of acute-phase proteins in goats. They found a significant increase only in the concentrations of Hp, but not in other acute-phase proteins. The changes of some inflammatory markers were evaluated also in goats around kidding [182]. Their results suggest that an increase of inflammatory indicators (mainly Hp) before kidding may be related to the changes in the energy balance status around parturition.

4.3.1.6. Ceruloplasmin

Ceruloplasmin (Cp) is a ferroxidase enzyme that is the major copper-carrying protein in the blood, and plays a role in iron metabolism [183]. Ceruloplasmin carries 70–95% of the total copper in plasma, and thus might play a role in Cu and iron homeostasis [184]. Furthermore, ceruloplasmin is involved in cellular prooxidant and antioxidant processes, and has antibacterial activities [185]. It is produced by the liver as apoceruloplasmin, an unstable non-copper-bound form, which subsequently reacts with seven copper atoms forming holoceruloplasmin, a functional and more stable product [186].

Ceruloplasmin has been evaluated as a marker of animal health and welfare [187]. Several studies in cattle indicate its diagnostic use with applications in many disease conditions, including uterine bacterial contamination, as well as clinical and subclinical mastitis [177, 188]. Hussein et al. [189] evaluated ceruloplasmin activity in dairy cows in different lactation stages, showing higher values in fresh-lactation stage. López-Alonso et al. [190] measured ceruloplasmin as a potential marker of hepatic copper accumulation in cattle. Studies in young animals have shown that the concentrations of ceruloplasmin in the serum increases during induced pneumonic pasteurellosis, with the highest concentration observed 2 and 4 hours after the inoculation [191].

4.3.2. The β-globulins

The β-globulins belong to group of globular proteins that migrate faster than γ-globulins in electrically charged solutions, but more slowly than α-globulins. The main components of the β-globulin fraction are transferrin and complement, which may correspond to the 2 subfractions (β_1 and β_2) identified in some animal species [81]. Other important proteins belonging to this fraction are: β_2-microglobulin, C-reactive protein, ferritin, hemopexin, plasminogen, and angiostatin. Furthermore, in response to the stimulation by different antigens, some IgM immunoglobulins may migrate in the β region, while the IgA and IgE immunoglobulins in the β-γ interzone, which may also correspond to the β_2 subfraction, identified in some animal species [5].

4.3.2.1. Transferrin

Transferrin (Tf), the iron-binding protein of serum has been described as a negative acute-phase protein. It is a strong chelator that is able to bind iron tightly but reversibly. The transferrin molecule has high affinity to bind two atoms of ferric iron (Fe^{3+}), being higher in the extracellular pH of 7.4 and decreases in the acidified endosomes, allowing the dissociation of Fe^{3+} [192]. The primary role of transferrin is to transport iron safely around the body to supply growing cells [193]. Essentially, all iron circulating in the blood normally is bound to transferrin. It renders iron soluble under physiologic conditions, prevents iron-mediated free radical toxicity, and facilitates transport into cells [194]. Similar to lactoferrin, transferrin inhibits multiplication and growth of certain viral, bacterial, and fungal organisms by iron inhibition.

Moser et al. [195] evaluated the concentrations of transferrin in cattle in various physiological states, in energy-deficient (ketotic) cows, in cases of several acute and chronic infections, as well as after the administration of endotoxins. The values of transferrin in healthy animals ranged from 2.0 to 6.6 g/l. While in animals with acute infections and ketosis, the values were in the range of 1.5 and 8.5 g/l, chronic infectious diseases (such as paratuberculosis) were associated with relatively low values (below 2 g/l). The evaluation of the effect of age on transferrin concentrations showed its lower values in adult animals compared to young animals [195]. Tóthová et al. [122] presented a marked increase of transferrin concentrations from day 7 of life, reflecting acceptable rate of protein synthesis and good nutritional status. Furthermore, the concentrations of transferrin increased in veal calves with iron deficiency above 8 g/l, resulting in negative correlation between hemoglobin and transferrin [195].

4.3.2.2. Lactoferrin

Lactoferrin (Lf), also known as lactotransferrin, is a multifunctional protein of the transferrin proteins capable of binding and transferring Fe^{3+} ions. Lactoferrin is a globular glycoprotein with a molecular weight of about 80 kDa, which shows high affinity for iron [196]. Although the overall structure of lactoferrin is very similar to that of transferrin, they differ in their relative affinities for Fe and the propensity for release of Fe [197]. The capability of lactoferrin to bind iron is two times higher than that of transferrin [198]. This bound is very strong and can resist pH values of as low as 4 [199]. The ability to keep iron bound even at low pH is important, especially at sites of infection and inflammation where, due to metabolic activity of bacteria, the pH may fall under 4.5 [200]. The most of bacterial pathogens necessitate Fe for metabolic activities, growth, and proliferation. Since lactoferrin has Fe-binding capacity, it reduces the growth of Fe-requiring pathogenic bacteria including enteropathogenic *E. coli* [201]. Lactoferrin is a major component of the innate immune system of mammalians and represents one of the first defense systems against microbial agents, which invaded the organism mostly by mucosal tissues [202]. It affects the growth and proliferation of many infectious agents including both Gram-positive and Gram-negative bacteria, viruses, protozoa, and fungi [203].

Lactoferrin is expressed in most biological fluids, including milk, saliva, and nasal secretions. It is present in blood, plasma, or serum in relatively low concentrations, but its concentrations increase during infection, inflammation, excessive intake of iron, or tumor growth [204]. Higher concentrations of lactoferrin were observed in bovine and human milk, or colostrum. The lactoferrin values in milk of healthy cows are quite variable and may range from 1.15 to 485.63 μg/ml. On the other hand, sub-clinical and clinical mastitis may lead to rapid increase of its concentrations positively correlating with SCC, stage of lactation, and milk yield [205, 206]. The concentrations of lactoferrin are higher in colostrum (varying between 1 and 5 mg/ml), during drying-off and early mammary involution period than during lactation [207, 208].

Lactoferrin plays a key role in the defense mechanisms of the mammary gland, contributing to the prevention of microbiological infection diseases [209]. Therefore, the concentrations of lactoferrin in milk are markedly influenced by the health status of the cows. Harmon et al. [210] induced *E. coli* infection in bovine mammary gland. In these cows, they found a 30-fold increase of lactoferrin values in the mammary secretion 90 h after the inoculation. Furthermore, they concluded that acute mastitis is associated with 30-fold increase of the concentrations of lactoferrin in the milk with the greatest production in the infected quarter.

4.3.2.3. C-reactive protein

C-reactive protein (CRP) was the first identified acute-phase protein, which was named according to its ability to bind to C-polysaccharide of Gram-positive bacteria [211]. It is a non-glycosylated protein from the group of pentraxins, and is composed of 5 subunits that firmly bind to C-polysaccharides [212]. Following bacterial infection, CRP binds to pathogen and activates the classical complement pathway leading to the opsonization of the bacteria

[213]. It also plays a role in the destruction of the infectious agent through the interaction with specific receptors on phagocytes, which may help in the reduction of tissue damage, and contribute to the tissue repair and regeneration [162].

There are considerable species differences in the magnitude and duration of changes in CRP concentrations during health disorders. In humans, dogs, and pigs, CRP is the major acute-phase protein with approximately 1000-fold increase in serum concentrations during acute inflammatory states [214]. In cattle, CRP has been reported to be a constitutive protein with only a minor increase during disease processes [7]. Despite this disadvantage, Schrodl et al. [215] evaluated the CRP concentrations in cows with mastitis, and found approximately 10-fold higher values in these cows (1083 ± 93 ng/ml) compared with healthy ones (82 ± 66 ng/ml). The data recorded by Lee et al. [216] showed a correlation between serum CRP concentrations and the health condition of dairy cattle.

4.3.2.4. Fibrinogen (in plasma)

Fibrinogen (Fbg), a precursor of fibrin, is also an acute-phase protein, which in coagulation cascade is the final substrate in the formation of a clot being converted to its insoluble fibrin form [217]. Fibrinogen belongs to the group of β-globulins and is present in the plasma. It is composed of three polypeptide chains linked by disulfide bridges and a glycoprotein [218]. Fibrinogen plays an important role in homeostatic processes, providing a substrate for fibrin formation. It is also involved in tissue reparation, and provides a matrix for the migration of inflammatory-related cells [219]. During an inflammatory reaction, fibrinogen can increase 2–3 folds, which may significantly increase blood viscosity and cause red cell aggregation, as well as may contribute to the growth of atherosclerotic plaques [220]. In human, studies showed an association between fibrinogen concentrations and subsequent cardiovascular disease risk, atherosclerosis, and acute thrombosis [221]. In cattle, fibrinogen has been used for many years to evaluate inflammatory and traumatic diseases, and is characterized by markedly increased synthesis in response to infection [222].

4.3.3. The γ-globulins

The γ-globulin fraction is predominantly composed of immunoglobulins (Ig) of various classes (IgG, IgA, IgM, IgD, and IgE). While in some animal species (cattle and goats) the γ-globulins constitute one overall fraction, in sheep, they may be visualized as two subpeaks: the γ_1 and γ_2 subfractions [104]. According to Kaneko [22], immunoglobulins from the γ fraction may migrate as fast or slow, which may be seen in these two subfractions. On the other hand, Vavricka et al. [76] indicated that some classes of immunoglobulins may migrate into the β-γ zone or β-region. Immunoglobulins (or antibodies) have major roles in the immune responses of the body, especially in response to foreign molecules, the so-called antigens. Their primary function is the protection of the host due to specific binding of one or a few closely related antigens in order to mediate their neutralization and elimination [223]. Immunoglobulins are produced by cells of the adaptive immune system, activated B cells and plasma cells, in response to the exposure to antigens [8].

http://dx.doi.org/10.5772/intechopen.72154

The immunoglobulins are glycoproteins composed of two heavy (H) and two light (L) chains linked by disulfide bridges [223]. According to the structure of the H chain, immunoglobulins are classified into the following classes: IgG, IgM, IgA, IgE, and IgD. The L chain consists of either kappa (κ) or lambda (λ) chain, which indicates the type of immunoglobulins. Based on the structural variations in the variable regions of H or L chains, immunoglobulins can be further divided into subtypes and subclasses. For example, two subclasses of IgG have been identified in cattle (IgG1 and IgG2) [7].

Most viral, bacterial, and toxin antibodies are of the IgG type and are present in all animals. It is the predominant type of immunoglobulins found in the body and has the longest serum half-life. IgE is involved in allergic and anaphylactic reactions, whereas IgA can be found in the secretions of the respiratory, genitourinary, and gastrointestinal tracts [7]. IgM functions be opsonizing antigens for destruction and fixing complement, and usually are associated with the first line of defense [224]. IgD is found in very low concentrations in the serum and has a short half-life. The functions of circulating IgD are not well understood [225].

5. Changes in the serum protein electrophoretic pattern

Several factors, including non-pathological and pathological conditions, may influence the concentrations of proteins in the serum, thus the entire profile of serum proteins [226]. Many disease processes are associated with abnormal serum protein profiles. Changes in the protein profile commonly occur as secondary symptoms in numerous diseases, but may be also the primary symptom of some specific disease conditions [227]. Thus, the results of the electrophoretic analyses of serum or plasma proteins may provide a basis for the establishment of further specific diagnostic procedures and may be helpful by the differential diagnosis of several disease processes. However, abnormalities in the serum protein profile must be interpreted with regards to many influences that are not associated with pathological processes.

5.1. Serum protein pattern variations related to non-pathological conditions

Variations in the serum protein profile and shifts in albumin and globulin concentrations may occur not only under pathological, but also under physiological conditions [102]. Animal age is one of these important factors that may affect the concentrations of the different serum protein fractions or their electrophoretic pattern [103]. It has been shown in young and adult cattle [228], where the most important age-related differences were observed in the α- and γ-globulin fractions. While the values of α_1-globulins were higher in calves, the adult animals had higher γ-globulin concentrations. In particular, it has been stated that the most important changes occur in the first month of the life of calves, and are associated with the changes in nutrition and adaptation processes during the neonatal period [229]. The total serum proteins and γ-globulin concentrations increase rapidly 1 day after the intake of colostrum, and then decrease gradually till the end of the first month of age. According to Hammon et al. [230], the concentrations of total proteins in the serum are very low at birth, due to the minimal quantities of immunoglobulins but, it increases during the first 24 hours of life as a result

of the intestinal absorption of proteins (particularly immunoglobulins) from colostrum. On the other hand, the concentrations of albumin decrease 1 day after colostrum intake, with a subsequent gradual increase from day 2 till the end of the first month of life. At birth, calve's alpha$_1$-globulins comprise almost 30% of total proteins, but their concentrations decreased approximately by 50% at 1 day after birth, with a further decrease up to day 30 of life [229]. In the absolute concentrations of α_1-globulins, a temporary slight increase after birth has been observed with a subsequent gradual decrease. The delivery is surely a stressful situation for the offspring and it could typically be expressed by higher concentrations of acute-phase proteins at birth, which migrate into this fraction [148]. The acute-phase response may be then substituted by the following increase of the IgG concentrations from the colostrum. Acute-phase proteins are produced mainly by the liver, which is less mature in newborn than in young or adult animals. Thus, the most of the acute-phase proteins have lower concentrations at birth than in the next days [231]. Similarly, large amounts of α-globulins were observed in lambs during the first month of life [232].

Pregnancy and lactation are further factors that may influence the concentrations of albumin and globulin fractions. Variations in the serum protein profile were found in ewes during the pregnancy and lactation, as well as in periparturient goats [233–235]. Changes in the concentrations of protein fractions during the last phase of pregnancy and early *post-partum* were recorded also in dairy cows [236]. Lower concentrations of total serum proteins were found by Grünberg et al. [237] in cows around parturition than outside the parturient period and in the following stages of lactation. These changes may be associated with the transfer of immunoglobulins from the bloodstream to the mammary gland for the synthesis of colostrum [238]. The results of Piccione et al. [235, 239] showed increasing values of α-globulins in dairy cows and ewes *post-partum*, which were probably related to the higher concentrations of the acute-phase proteins in response to the processes occurring around the time of parturition.

The concentrations of serum proteins may be influenced also by hormonal changes and stress. Stress may cause a decrease of serum protein and albumin concentrations, but often may be accompanied by an increase of the α_2-globulin fraction associated with the acute-phase response [7].

5.2. Pathological serum protein pattern: dysproteinemias

A wide variety of diseases can cause changes in the serum protein pattern [240]. The serum protein electrophoresis is a very important technique for the evaluation of these abnormalities and the nature of the hyperproteinemia or hyperglobulinemia [241]. The protein electrophoresis may be very useful when routine investigations are not effective for making medical decisions, providing the basis for further specific laboratory analyses [22, 242].

5.2.1. Changes in the albumin fraction

The decrease of the concentrations of albumin is one of the most frequently occurring types of dysproteinemias. Hypoalbuminemia can be caused by decreased production due to liver diseases such as chronic hepatitis, cirrhosis, or liver failure [243]. Hypoalbuminemia may be

also present in renal diseases and nephrotic syndrome, in which there is an increased loss of this protein in urine caused by glomerular damage [244]. Moreover, low albumin concentrations may indicate chronic malnutrition, inadequate protein intake, or being associated with gastrointestinal diseases, internal parasitism and protein losing enteropathy [245]. On the other hand, serum albumin is the major negative acute-phase protein and its synthesis may be markedly reduced during the acute-phase response [246].

Rarely, a serum protein anomaly called bisalbuminemia may be observed on the electrophoretogram. Bisalbuminemia is characterized by the occurrence of a bicuspid electrophoretic pattern in the albumin fraction, where albumin produces two heads (equally staining bands or bands of unequal intensity) [247]. In this abnormality, albumin may either have increased (fast type variants) or decreased electrophoretic mobility (slow type variants) [248]. In humans, the presence of bisalbuminemia have been described in some pathological conditions, including chronic renal diseases, nephrotic syndrome, diabetes mellitus, pancreatic disease or Alzheimer's disease [249]. In ruminants, bisalbuminemia was not yet found. According to Vavricka et al. [76], the presence of bisalbuminemia may be caused by increased mobility of albumin due to its binding to bilirubin, non-esterified fatty acids, penicillin or acetylsalicylic acid.

The increased concentration of albumin in the serum is called hyperalbuminemia, which may be observed in cases of severe dehydration. However, hyperalbuminemia was recorded also in dogs with hepatocellular carcinoma [250].

5.2.2. Changes in the globulin fractions

Increases in the globulin fractions may be frequently seen on serum protein electrophoretograms. Since many acute-phase proteins belong to the alpha-globulin fraction, increase in the α_1- and α_2-zones may be typical for many acute, as well as chronic inflammatory diseases caused by the activation of the host inflammatory responses [71]. Increased α-globulins (predominantly α_1-globulins) were found in sheep naturally infected with *Babesia ovis*, as well as in calves affected by respiratory diseases [251, 252]. The α_2-globulin fraction typically increases in patients with nephrotic syndrome as a result of the increased synthesis of α_2-macroglobulin that migrates in this fraction. Because of its size, the α_2-macroglobulin is unable to pass through glomeruli and therefore it remains in the bloodstream [253]. Decreases in the α_1-globulin fraction may be detected in the α_1-antitrypsin deficiency, a rare genetic disorder in humans and even more rare in animals, but in ruminants, it was not yet detected [254]. Similarly, the α_2-globulin zone may be typically decreased in hemolytic anemia, when haptoglobin from this fraction binds with the free hemoglobin released from the destroyed red blood cells, forming haptoglobin-hemoglobin complexes that are rapidly removed by phagocytes [76]. On the other hand, the inflammatory conditions that develop in association with hemolytic anemia leads to an increase of haptoglobin concentration that may induce an increase of α_2-globulins [255].

Some acute-phase proteins migrate into the β-region. Thus, several inflammatory diseases and infections may be accompanied also by increases in the β-fraction as a result of the elevated

production of these proteins. Kaneko [22] stated that increases solely in the β-globulin fraction are not frequent and may be typical for active hepatitis. Chronic persistent liver disease, liver cirrhosis, as well as nephrotic syndrome may be associated with elevations in the β-region due to the increase of the concentrations of β_2-microglobulin in these conditions [256]. High β-globulin concentrations may be associated also with hypercholesterolemia, which is caused by increased concentrations of β-lipoproteins in this fraction [257]. Furthermore, increased β-globulins are typical for iron deficiency anemia associated with higher values of transferrin [258]. The increase of β-globulins in hemolytic anemia may depend on the presence of free hemoglobin that typically migrates in this region. On the other hand, malnutrition is often accompanied with decreased concentrations of β-globulins.

In some conditions, the increase in the β_2- and γ-globulin fractions may result in a beta-gamma fusion. This phenomenon is called β-γ bridging and is characterized with no clear demarcation between these two fractions. It is caused by an increase of the concentrations of IgM or IgA, which may migrate in the region between the beta and gamma zones [259]. According to some authors, the pattern of β-γ bridging is pathognomonic for chronic liver diseases or hepatic cirrhosis [260]. However, Camus et al. [261] stated that β-γ bridging does not have a strong predictive value for hepatic diseases in some animal species, including dogs, cats, or horses, and may be frequently found in association with infectious diseases, including leishmaniasis or ehrlichiosis [262]. Tóthová et al. [263] observed also a β-γ fusion in cows with severe hoof diseases. Other possible source of the β-γ bridge is the use of plasma instead of serum, caused by the migration of fibrinogen between the β and γ regions [16].

Increases of the γ-globulin fraction (the so-called gammopathies) belong to the frequent serum protein alterations, and are typical for many pathological conditions. Two types of gammopathies were differentiated: monoclonal and polyclonal. Monoclonal gammopathy is characterized by a sharp, homogenous, spike-like peak in the focal region of the γ-globulin zone. This pattern may be caused by the production of excessive amounts of one type of immunoglobulin secreted by a single clone of B lymphocytes, or an immunoglobulin fragment described as paraprotein or M protein [264]. Multiple myeloma is the most common malignant disorder of plasma cells, in which usually IgA and IgG paraproteins can be found [265]. Monoclonal gammopathies in farm animals are not frequent. Some cases were recorded in horses and small animals, which has been associated with plasma cell myeloma, malignant lymphoma, or erythrophagocytic multiple myeloma [264, 266].

Polyclonal gammopathy is associated with the presence of a diffuse hypergammaglobulinemia, in which all immunoglobulin classes may be increased. It is characterized by a diffuse, broad increase in the γ-globulin zone on the electrophoretogram. This swell-like elevation of γ-globulins is mostly caused by inflammatory reactions, and usually indicates a non-malignant condition [71]. The most common causes of polyclonal gammopathies are chronic inflammatory processes (gastrointestinal, respiratory, endocrine, cardiac), severe infections, as well as immune-mediated disorders [76, 267]. The decrease of the concentrations of γ-globulins in the serum is called hypogammaglobulinemia. This pattern is typical for fetal or precolostral sera in some animal species. In calves, precolostral serum normally contains no (agammaglobulinemia) or very low concentrations of γ-globulins, but they start to increase within a few hours

http://dx.doi.org/10.5772/intechopen.72154

after the intake of colostrum, and the absorption continues for up to 24–36 hours after birth, after which gut permeability ceases [268, 269]. Hypogammaglobulinemia may be commonly seen also in patients with recurrent infections or in cases of immune deficiency, including primary immunodeficiency disorders [1].

The aforementioned shifts in the concentrations of albumin and globulins lead also to changes in the albumin: globulin ratio (A/G). The normal A/G ratio is in the range of 0.6–0.9 in cows, but the relative concentrations of albumin and globulins may be altered in many disease conditions, which results in changes in their proportion [22]. Decreased A/G ratio may be associated with the overproduction of globulins, decreased synthesis of albumin, or with losses of albumin from the circulation. On the other hand, higher A/G ratio is usually caused by the underproduction of globulins. Thus, the interpretation of A/G ratio is very important itself providing information about the changes in pattern of serum proteins, and could help in the classification and identification of dysproteinemias [105].

6. The use of serum protein electrophoresis in bovine clinical practice

The analysis of serum proteins and their electrophoretic separations have been extensively used in human medicine for many years. Serum protein electrophoresis has been studied intensively also in small animal and equine medicine, especially to support a clinical diagnosis of diseases characterized by dysproteinemia (leishmaniasis, ehrlichiosis, feline infectious peritonitis), or to identify the presence of inflammation with increased α-globulins [76]. In bovine clinical practice, serum protein electrophoresis is a rarely used diagnostic tool.

The diagnostic significance of protein electrophoresis in cows with traumatic pericarditis was evaluated by Yoshida [270]. In the affected cows, slight hypoproteinemia, moderate hypoalbuminemia, and a slight increase of the α- and β-globulin concentrations were observed. In cows with purulent pericarditis, they found a tendency of hypergammaglobulinemia, while fibrinous or sero-fibrinous pericarditis was associated with a large indentation between the β- and γ-fractions. The changes in the electrophoretic pattern of serum proteins and immunoglobulin concentrations were studied also in cows with lymphoma [271]. Moderately increased concentrations of α_2-globulins were found in these cows, while the β_2-globulin fraction was significantly decreased due to the lower concentration of immunoglobulins. Recently, Tóthová et al. [252] evaluated the effect of chronic bronchopneumonia on the serum protein pattern in calves. These authors found significantly higher concentrations of α_1-, β_2-, and γ-globulins in the affected animals compared with healthy ones. Alterations in the electrophoretic pattern of serum proteins were found also in dairy cows with inflammatory diseases [263]. In this study, *post-partum* metritis was associated with significantly lower concentrations of albumin and higher values of α_1-globulins compared with clinically healthy cows. The cows with clinical mastitis showed higher β_1- and γ-globulin fractions, while in cows affected by hoof diseases significantly lower concentrations of albumin and higher values of α_1-, β_1-, β_2-, as well as γ-globulins were found. Furthermore, the serum protein electrophoretic pattern of more than

half of the group of cows with hoof diseases showed β-γ bridging [263]. Constantin et al. [272] evaluated the serum protein profile and its changes in cows affected by clinical endometritis, and found lower concentrations of albumin and higher values of α_2-, as well as γ-globulins.

7. The use of serum protein electrophoresis in small ruminants

The usefulness of the electrophoretic separation of serum proteins was studied by Woolf et al. [273] in bighorn sheep with chronic pneumonia attributed to *Mycoplasma*. In this study, diseased sheep had significantly lower albumin, and higher α_1- and γ-globulins. The alterations of the serum protein electrophoretic profile were investigated also in sheep naturally infected with *Babesia ovis* [251]. In this study, the diseased sheep before treatment had markedly lower concentrations of both total serum proteins and all protein fractions when compared with healthy animals. A significant increase of total serum proteins and globulins (except for the α-globulin fraction) was found 5 days after treatment, but the values were still lower than those obtained in healthy sheep. The aforementioned authors stated that babesiosis may induce intense proteolysis of the circulating proteins probably due to the altered protein synthesis by the liver, which was improved by the eradication of parasites. Similarly, the alterations in the serum protein profile induced by the infection with *Haemonchus contortus* were studied by Diogenes et al. [245] in goats. In the infected goats, severe hypoproteinemia and hypoalbuminemia were observed, while the concentrations of α- and γ_2-globulins were markedly increased. Experimental infection of goats by *Fasciola hepatica* resulted also in changes in serum protein profile, manifested by decreased concentrations of albumin, increased values of total serum proteins, γ-globulins, and increased proportion of acute-phase proteins from the α- and β-globulin fractions [274].

Changes in the serum proteinogram were found also in sheep with acute ruminal lactic acidosis with the most intense alterations in the α-globulins [275]. Increased concentrations were recorded in the concentrations of haptoglobin, probably due to the death of Gram-negative bacteria caused by decreased ruminal pH, as well as inflammatory processes induced by ruminitis. Acute ruminal acidosis in sheep was accompanied also by the increase in the values of α_1-antitrypsin, ceruloplasmin, as well as fibrinogen.

8. Conclusions

The obtained data suggest that the analysis of serum protein profile may be a useful diagnostic tool also in ruminants. It may provide important diagnostic information for clinicians in the determination and differentiation of dysproteinemias or paraproteinemias. Changes in serum proteins can be indicative of many health problems and may serve as potential diagnostic markers for some pathological conditions. The abnormal electrophoretic pattern of serum proteins may be characteristic for some disorders or disease conditions, but others may indicate only non-specific pathological processes. Despite of this low specificity in the diagnosis of some diseases, the determination of the serum protein pattern also in ruminants and

the correct interpretation of their results are very useful for clinicians in diagnosing healthy and sick animals, and may provide a basis for further specific laboratory investigations. This review suggests that the analysis of serum proteins in ruminants provides still many areas to study their changes in various health disorders and diseases.

Acknowledgements

This work was supported by Scientific Grant Agency of Ministry of Education SR No. 1/0154/15 and 1/0486/17.

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Edited by Muhammad Abubakar

It is very essential to understand the recent advances in ruminant science to recognize and control diseases and disorders in these animals. Our book, *Ruminants - The Husbandry, Economic and Health Aspects*, provides a concise introductory chapter and details about the main aspects of ruminants' science and production. This is the first edition of the book, so it covers the introductory level of topics, which are written specifically for veterinary students, classroom use, and practitioners who require more knowledge of dairy animal health and production. The book covers an introductory chapter and sections on husbandry and economics as well as animal health. Each book section comprises chapters from renowned experts from the area and gives readers a unique opportunity to explore the topic.

Published in London, UK

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ISBN 978-1-83881-336-9