Chapter V: Materials and Methods

Section 1. Experimental design

1.a) Animals and study area

The study was carried out on a sheep farm located in the township of Zeri (MS) in the Lunigiana area in the locality of Piagna, at an altitude of 900 meters a.s.l. The entire flock of Zerasca sheep is composed of approximately 200 animals of which only a small number originate from cross-breeding with Sardinian sheep, kept only for cheese-making and for the nourishment of lambs in case of necessity.

The sheep are raised on six hectares of land rented by the shepherd, and several more hectares of pastures which are public lands, also named “agrarian communities” (Ronchi and Nardone, 2003). Animals are raised following an extensive management system where they are kept almost all year round on open pastures and grasslands. Sheep nourishment consists of grass, shrubs, bushes and other plants they come across during grazing on marginal lands and in forests.

Grasslands and pastures are managed without employing chemical fertilizers or any particular agronomical techniques, except for the use of organic manure which is regularly spread on soil.

Old stone houses are often used as barns for sheltering the flock during harsh climatic conditions. The animals’ diet is based on natural pasture with corn supplementation provided in summer and winter. The proprietor does not cultivate crops therefore acquires the necessary hay stacks and nutritional supplements.

Fresh water is supplied by drinking troughs placed inside barns and on open fields.

Females are covered at around 18 months of age whilst males begin their reproductive activity at approximately 9 months. The ewes’ career lasts ten years whilst rams are culled at two years of age.

Lambs are destined for the production of the local and typical Zeri heavy lamb, which is obtained slaughtering lambs of 2-3 months of age at a body weight of 20-25 kg.

Milk obtained from the Sardinian cross-breeds is used for the manufacture of ovine cheeses which is carried out in a personal laboratory.

In addition to the main activities, the breeder is also involved in the production of the characteristic sheep wool “Mezzalana” which is transformed into fabric and sold.

The control of endoparasites is normally brought about using pharmacological treatments but before the beginning of this study the animals did not receive any treatment for over a year and homeopathy was never applied.

1.b) scientific protocol

Twenty-seven adult female sheep were casually selected from the entire flock and randomly divided into three groups. In order to verify the homogeneity of the groups randomly chosen, parasitological analysis of the faeces of all selected ewes was performed. From
January 2009 to October 2009, fecal and blood samples were collected from the 27 naturally infected adult ewes.

The values of the FECs were grouped into four levels of infection where Level 1 corresponds to 0 EPG; Level 2 ranges from 1-300 EPG which is still an acceptable degree of infestation; Level 3 ranges from 300-600 EPG where it is generally recommended to monitor closely animals because the probability of reaching Level 4 is high. Level 4 corresponds to an excretion of more than 600 EPG where the animals’ health status is critically at risk. Subsequently, a distribution was made of each group in order to verify the percentage of animals in each level. This way, the gravity of infestation is also evaluated.

The three experimental groups were each made up of 9 animals: (i) the untreated group (“Control”); (ii) the chemically treated group (“Drug”) and the homeopathically treated group (“Homeopathy”). Each animal was identified individually by applying a coloured ear tag. During the experimental period four fecal sampling were taken in the months of February, April, July and October. Samples were collected directly from the rectal ampoule and individually examined using the Concentration McMaster Technique (Permin and Hansen, 1998) to estimate the Fecal Egg Count of gastrointestinal nematodes. Two blood sampling were performed: the first at the beginning of the trial and the second after one month from the administration of the treatments. Samples were taken from the jugular vein using Vacutainer® test tubes in order to analyze hematological, metabolic and immunological parameters.

During the first and last samplings, FAMACHA scores and Body Condition values were appointed to each animal and registered. In addition, the investigation also aimed at identifying ewes that were in their periparturient period which ranges from 30 days before and 30 days after parturition.

During this period the animals of the three groups were maintained in the same environment conditions.

1.c) Chemical drug treatment

The treated group received the benzimidazole drug Netobimin Hapadex® 5% (Schering-Plough) once during the month of March. The pharmaceutical company recommends that 7,5 mg of Netobimin be administered orally per kilo of live weight for the treatment or prevention of ovine gastrointestinal or pulmonary strongyles. On the other hand, 20 mg/kg should be administered when treating against cestodes (tapeworms) and trematodes (liver flukes). Mathematical calculations were performed to determine the precise quantity of drug to administer in order to treat against all the mentioned parasites which was found to be 4 ml/10kg of live weight. Therefore 20 ml of Hapadex® 5% were administered to each animal. Moreover, the pharmaceutical company orders that the milk and meat obtained respectively 72 hours and 21 days later must not be sold.

Netobimin is transformed into Albendazole in the gut which is thought to be the true anthelmintic substance. Curiously, Netobimin is effective against the hypobiotic larvae of Ostertagia ostertagi and when administered orally, maximum concentration is achieved within 7 hours.
1.d) Homeopathic remedy

To the animals of the homeopathic group three ml per individual of *Lachesis mutus* XMK (10,000K Korsakov dilution), diluted and potentized in mineral water, were administered once a day for three consecutive days. Afterwards, the remedy was administered once every two weeks for 2 months, and then once a month until the month of June.

The remedy was kindly supplied from the company C.e.m.o.n.-UNDA.

The homeopathic medicine was chosen by a specialized veterinarian who completed the homeopathic examination and collection of anamnestic data of the flock, in order to identify the general symptoms present. The homeopathic examination is accomplished thanks to a brief interrogation of the stock-holder and a thorough clinical inspection of the animals. The general goal is to identify the typical and more peculiar responses or behavioural characteristics the animals manifest, with regard to the physiological and pathological aspects belonging to the physical, mental and emotional systems.

Analysis of the observed and recorded symptoms, has the goal of finding a possible association between them. Clinical-case analysis in homeopathic medicine must be detached from the more symptom-specific approach practitioners apply in conventional medicine. Undoubtedly, the approach in choosing the remedy is a holistic one where nutritional, environmental, ethological, sanitary and managerial aspects must be taken into consideration (Pisseri *et al*., 2001). In searching for correlations between the observed symptoms, one is actually discovering the true reactivity of an individual and pointing out the weak points of the global psycho-somatic entity (Mangialavori and Marotta, 2004).

The homeopathic repertory is a database which groups together the experimental and clinical homeopathic results. The symptoms are therefore compared to the registered effects of each remedy listed in the repertory, in order to select the one which explicates the most similar effects (Benvenuti *et al*., 2008). The remedy prescribed to the entire flock is selected on the basis that individuals of a flock are quite homogeneous among themselves from a genetic, ethological, nutritional and sanitary point of view. The chosen remedy is thus prescribed at a given dilution and route of administration.

**Homeopathic repertorization of the remedy:**

The repertorization performed resulted in the selection of *Lachesis mutus*, which is a substance derived from the homonymous venomous snake belonging to the Crotalinae subfamily, based on the similarities with the behavioural and physiological aspects of the sheep studied.

The ten-millionth Korsakov dilution was chosen in order to reduce the number of administrations necessary performed by the flock-holder all the while abiding by the protocol correctly.

At the beginning of the study, the clinical homeopathic examination performed on the animals underlines the uniform behaviour of the flock all animals are quite calm and show signs of curiosity towards humans.

The sheep are lively and dynamic; sometimes even unsubmissive and disobedient in responding to the shepherd’s commands, manifesting their own will and pride.
Results collected from the pathologic anamnesis highlighted some episodes of acute mastitis, a few cases of respiratory problems with coughing, and a low mortality rate among lambs which was caused by the respiratory problems.

Reportorial symptoms:
MIND; QUIET; disposition
MIND; VIVACIOUSNESS
MIND; LOQUACITY
CHEST; INFLAMMATION; Bronchial tubes, bronchitis
RECTUM; WORM, WORMS; complaints

<table>
<thead>
<tr>
<th>Total Rubrics</th>
<th>Total FAMILIES</th>
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Figure 1. Repertorization analysis (Mac Repertory 5.5).

The symptoms listed above were entered in the automated software for the repertorization analysis and the selection of the suitable remedy. *Lachesis mutus* is the remedy which has the highest number of similar effects with the specific symptoms (see Figure 1.)

1.e) Parasitological techniques

The Concentration McMaster technique is slightly more sensitive than the simple one where 20 eggs per gram of faeces can be recovered. This method requires a centrifuge but is recommended when many samples must be handled simultaneously.

The procedure dictates to weigh out 4.0 g of faeces and to transfer them to a labeled beaker. 56 ml of tap water were added using a measuring cylinder so that the ratio remains 14 ml of tap water for every gram of faeces, ensuring that every 15 ml of suspension correspond to 1.0 g of faeces. With a stirring device the faeces are mixed thoroughly. The fecal suspension
is allowed to rest for 30 minutes at room temperature, followed by another thorough mixture using the device.

At this point, the hard clumps of faeces should be completely dissolved. The fecal suspension is poured through a tea strainer into a second beaker after having discarded the retained debris and stirred properly. After filtering, the fecal suspension was poured into a test tube to the 10 ml mark. These 10 ml represent 2/3 of 1.0 g of feces.

The test tube is placed in a centrifuge for 5-7 minutes at 1200 RPM (revolutions per minute). The supernatant is carefully eliminated with a pipette avoiding resuspending the sediment. In this way, the sediment still represents 2/3 of 1.0 g of feces. Now it is possible to add flotation fluid, unless the procedure needs to be interrupted and the test tubes are refrigerated for storage (up to a maximum of 7 days). Flotation fluid is added up to the 4 ml mark which represents 2/3 of 1.0 g of feces. With a Pasteur pipette, the sediment is sucked up and down in order to resuspend it without making bubbles. Immediately after, both sides of the McMaster counting chambers are filled with the fecal suspension.

The McMaster chambers are left to rest for another 3-5 minutes and subsequently counted in both counting fields under the microscope at a 4 × 10 magnification. Nematode eggs and coccidia oocysts are counted separately. The fecal egg count is calculated by multiplying the number of eggs counted by 20 (Permin and Hansen, 1998).

**1.f) Procedures used for blood samples**

During the field trial, two blood samples were drawn from each animal; the first in the beginning of March and the second in the month of April. Blood samples were always drawn in the morning, around nine hours after the last meal consumed.

All samples were examined in the laboratories of the ‘Istituto Zooprofilattico Sperimentale delle Regioni Lazio e Toscana’ situated in Rome. The samples of 10 ml were collected using suitable test tubes. One sample of blood was destined to be used for the full blood count and blood smears whilst the other was used for the blood chemistry tests.

The samples of blood preserved in K3EDTA test tubes were refrigerated, not frozen, during transportation and sent within 6 hours after drawing the blood. The complete blood panel was obtained by using the CELL-DYN 3500® automated hematology analyzer. Four methods of analysis are employed for the measurement of haematological parameters:

1. optic flow cytometry for the measurement of WBC counts (WOC) and leukocyte differential count. This methodology is referred to as the Multi-Angle Polarized Light Scattering Separation (MAPSS). Sensors placed at different angles from the cells are responsible for determining the radius of the laser beam deflected off the cell surface once it hits it. A certain radius corresponds to a certain cell morphology and thus a certain cell type;

2. electrical impedance channel for the total white blood cell count (WIC) after dilution with a lytic reagent, which should confirm the WOC value;

3. another impedance channel provides all information regarding fragile lymphocytes and hypotonically resistant red blood cells, in addition to erythrocyte and platelet count;
4. the dosage of hemoglobin is effectuated by spectrophotometry based on the colorimetric determination of hemoglobin released after using a red blood cell lyzing reagent.

This particular apparatus is able to measure, count and calculate hematological indexes and is calibrated for veterinary usage. The quantity of blood sucked from the test tube is of 130 μl ± 5% and is separated into three equal quanta which are then diluted for analysis (Archetti and Ravarotto, 2002).

In addition, leukocyte differential analysis is also carried out manually using blood smears for more accurate results.

Hematocrit is evaluated by using a particular centrifuge capable of fulfilling rotations at a velocity of 12,000 RPM for 1-3 minutes. The capillary tubes contain the blood sample and after centrifugation they are positioned on a layer measuring device with which red cell and buffy coat constituent band heights are measured. There are three singular levels which derive from centrifugation:

- plasma (top level) which is of a yellowish colour and represents 55% of total blood
- buffy coat (<1% of total blood) is made up of platelets, leukocytes and nucleated erythrocytes which are in the lowest zone given their superior weight in density
- red fluid (45% of total blood) which is made up of enucleated red blood cells (Lubas, 2004).

A measuring device is used to quantify the Hematocrit value by placing the capillary tube on the scale printed thereon. A cursor is moved from left to right until the upper line of the scale coincides to the highest level reached by plasma. In this way, the intersection on the scale between plasma and the red fluid level points out the value of PCV in percentage (Lubas, 2004).

Furthermore, the neutrophil/lymphocyte stress factor (NLSF) ratio was calculated and considered as a parameter which effectively reflects the inflammatory/immune response (Zahorec, 2001).

Chemical screening is achieved by putting the blood sample in centrifugation at 4°C for 20 minutes at 3000 RPM. Plasma is thus obtained and frozen at -20°C in two equal quantum of at least 1 ml for each sample. Before analysis, plasma must be defrosted at 37°C for 10 minutes. The comprehensive metabolic panel includes the following parameters and method principles:

- Alanine amino transaminase (ALT) (IFCC, without P5’P – blood without anticoagulant);
- Aspartate amino transferase (AST) (IFCC, without P5’P – blood without anticoagulant);
- Blood Urea Nitrogen (BUN) (Enzymatic UV (urease enzyme solution)) – blood without anticoagulant;
- Cholesterol (CHO) (CHOD PAP = enzymatic hydrolysis and oxidation) – blood without anticoagulant;
- Cortisol (CORT) (Radio-immunology using radio-isotopes) – blood without anticoagulant;
- Creatinine (CREAT) (Colorimetry (Jaffe’)) – blood without anticoagulant;
Glucose (GLU) (Enzymatic UV (Hexokinase)) – blood without anticoagulant;
Non-essential fatty acids (NEFA) (Colorimetry) – blood without anticoagulant;
Blood proteins (PROT) (Biuret test) – blood without anticoagulant;
Triglycerides (TRIG) (GPO,PAP) – blood without anticoagulant.

The determination of lysozyme and BPI are obtained from the serum thus from the sample of blood preserved without an anticoagulant and subsequently centrifuged in the laboratory. Centrifugation is accomplished for 20 minutes at 2500 RPM at 4°C. Serum must be divided into four equal quanta and then stored at -80°C if the tests cannot be run immediately (Archetti and Ravarotto, 2002). For the evaluation of lysozyme and BPI, blood is submitted to a microbiological examination.

The measurement of CD4+ and CD8+ are achieved using the sample of blood preserved with an anticoagulant and analyzed via flow cytometry.

1.g) Zootechnical parameters

The previous parameters examined are able to describe not only the health status of the animals but also provide indirect information regarding animal welfare. In this opinion, reproductive performance and productivity were evaluated through the assessment of three indicators such as Body Condition Score, FAMACHA® scores and Fertility rates of the studied sheep.

Body condition Score:

BCS was first introduced by Edmunsson as an important figure in understanding problems present in a flock. Infact, this method can help veterinarians in clinically approaching an entire group of animals as opposed to the singular individual. BCS indicates the amount of subcutaneous adipose tissue a farm animal possesses which in metabolic terms, corresponds to the reserve of energy. A scale of numbers is used in appointing the score which goes from 1 to 5 with intervals of 0,25 decimal places: 1 corresponds to emaciation whereas 5 is considered obese (Tolasi and Ruggeri, 2008).

BCS is a reliable indicator of sheep metabolic welfare and it should never be below 1,5 or above 3,5 (Sevi et al., 2009). Low BCS depends on a on an intense mobilisation of body fat reserves (and to a lesser extent of nitrogen reserve), due to reduced energy intake or increased energy output, which mainly occur under high heat load situations; during suckling and early stages of lactation. Apart from sickness, deviations from average body condition scores depend on inadequate feeding management in terms of excessive or limited energy content of the diet and of unbalance between nutrient intake and requirements of the animal on a given physiological stage (Caroprese et al., 2009).

FAMACHA®:

FAMACHA is an acronym of the name of the originator of this system, Dr. Faffa Malan (FAffa MAlan CHArt) (Di Loria et al., 2009). The FAMACHA® system was validated to manage H. contortus-infected small ruminants in the United States but was subsequently adopted in South
Africa as a method of classification based upon state of anemia (Burke and Miller, 2008). The colour of the lower eyelid mucous membrane of each animal is classified into five categories as a morbidity marker for Haemonchosis according to the FAMACHA eye colour chart: 1=red, non-anemic; 2=red-pink, non-anemic; 3=pink, mildly anemic; 4=pink-white, anemic; 5=white, severely anemic. FAMACHA scores can be expressed up one decimal place (Burke et al., 2007). The FAMACHA system has been validated as a method for identifying anemic animals therefore on the basis of these findings, only severely infested animals can be dewormed using this simple tool, thereby saving dewormer and preserving more worms in refugia (Burke et al., 2007). The tricky part is finding the threshold under which all animals must be treated - eye scores ≥3 versus ≥4. For this goal, PCV values can be considered seeing as they provide more accurate information regarding the state of anemia (Burke et al., 2007).

Burke et al. (2007) established that the relationship between FAMACHA scores, PCV and FEC counts were indeed significant. They found that in treating animals with eye scores of 3 or 2 as opposed to animals with 4 or 5, a few non-anemic animals were treated thus increasing the usage of dewormer. However, leaving untreated truly anemic animals is far worst. Conclusively, eye score 3 represents the best threshold. It is also underlined that this system should not be the sole criterion to decide whether to drench or not, but should be used together with other indices such as BCS, PCV and FEC, all found to be profoundly correlated (Burke et al., 2007).

The FAMACHA system has been used in another occasion as a tool in identification of sheep with parasite resilience or resistance. Knowing that in resilient animals PCV values and FAMACHA scores denote several differences because the individual is able to resist anemia during high FECs, but will not be identified by the FAMACHA system in cases where they are not apparent, it is probable that resilience as opposed to resistance is preponderant. In these specific cases, it is indicated to consider FEC values which allow detection of those resilient animals but not the resistant ones. However, if one were to select only the resilient animals for drenching, animals with physiological PCV values and high FECs (the more susceptible ones) would inevitably be left untreated and able to contribute to pasture contamination, and even die seeing how often these are the younger animals (Burke and Miller, 2008).

Fertility:
Flock fertility was estimated based on farm reports.

1.h) Statistical analysis
The values resulted from FECs were divided into 4 levels in order to describe the severity of worm challenge. Level 0 corresponds to 0 egg counts; Level 1 corresponds to FECs between 1 and 300 which is still an acceptable level of egg counts; Level 2 falls between 300 and 600 which corresponds to the range of eggs which should be closely monitored because this could lead to zootechnical damage and moreover, animals could reach Level 3 (more than 600 EPG), where health conditions are highly endangered.
Statistical research was accomplished by the analysis of variance where variability factors in the model used were two: the date of sampling and the group nested into the date of sampling.

All data regarding EPG and OPG were transformed logarithmically with the following formula to normalize error (Baker et al., 1997):

$$ y = \log (EPG + 25) $$

All other non continuous data regarding blood parameters was transformed using logarithm_{10} in order to normalize the variance.

The one-way analysis of variance was carried out by using the different groups as factors of variability. The first sample represents the health status of untreated animals which renders the differences found between means of the first and second sampling evident. This statistical model adopted shows a significant effect of the group factor nested into the date of sampling.

Correlations were estimated between EPG, Hgb, PCV, blood proteins and triglycerides using Pearson’s correlations.

Analysis of variance was performed by including all transformed data into the JMP Software, 5.0 computed version of SAS Institute (2002).