

UNIVERSIDADE DE LISBOA  
FACULDADE DE MEDICINA VETERINÁRIA

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COMPARATIVE STUDY OF THE HEALING PROCESS OF DISBUDDING WOUNDS USING  
BEPANTHENE® OR CYCLOSPRAY®

GABRIELA DIAS MARTINS

ORIENTADOR:

Doutor George Thomas Stilwell

2022

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GABRIELA DIAS MARTINS

DISSERTAÇÃO DE MESTRADO INTEGRADO EM MEDICINA VETERINÁRIA

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2022

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Título da Tese ou Dissertação: Comparative study of the healing process of disbudding wounds using Bepanthene or Cyclopray

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Faculdade de Medicina Veterinária da Universidade de Lisboa, 7 de Julho de 2022

Gabriela Dias Martins

(indicar aqui a data da realização das provas públicas)

Assinatura: \_\_\_\_\_

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Lastly, I would like to thank every veterinarian, student and nurse that took part in this study and made this possible.

## Resumo

### **Estudo comparativo do processo de cicatrização de feridas de descorna com o uso de Bepanthene® ou de Cyclopray®**

A descorna é um procedimento comum nas explorações leiteiras, beneficiando o manejo, transporte e bem-estar animal. O método mais utilizado é a termocauterização, responsável por queimar o tecido que gera o corno. Após a intervenção, geralmente, as feridas resultantes são tratadas com antibiótico sob a forma de spray. Atualmente, a resistência a antibióticos está associada a uma elevada morbidade e mortalidade, tanto em medicina humana como em medicina veterinária, devendo investir-se na monitorização do uso de antibióticos, novas políticas e desenvolvimento de tratamentos alternativos em nome de Uma Só Saúde. O objetivo deste estudo prospetivo é a minimização do uso de antibióticos em animais de produção, recorrendo a formas alternativas de tratamento de feridas de descorna. O Bepanthene® (Dexpantenol) surge como opção, tratando-se de um produto amplamente usado em medicina humana no tratamento de irritações e queimaduras da pele.

A comparação entre o processo de cicatrização de feridas de descorna, tratadas com Bepanthene® ou Cyclopray® (Clortetraciclina), foi alcançada através da apresentação de fotografias das lesões a um painel de avaliadores, constituído por sete médicos veterinários, cinco estudantes de medicina veterinária e cinco enfermeiros da área da medicina humana. Para realização da classificação das lesões, o painel aplicou um formato adaptado de uma escala de cicatrização certificada, Bates-Jensen Wound Assessment Tool, constituída por sete parâmetros, que culminam num valor total posteriormente utilizado na análise estatística.

Na avaliação dos médicos veterinários, verifica-se uma diferença estatisticamente significativa (valor  $P < 0.05$ ), entre os dois produtos, para o parâmetro “Bordos”, indicativo da superioridade do Bepanthene®, em relação ao Cyclopray®, nesta vertente da cicatrização. A classificação dos estudantes mostrou diferenças estatisticamente significativas, entre os dois produtos, para os parâmetros “Tipo de Exsudado” e “Quantidade de Exsudado”, valorizando a ação do Bepanthene® nos parâmetros mencionados. A avaliação apresentada pelos enfermeiros não mostrou qualquer diferença estatisticamente significativa entre os produtos para qualquer um dos parâmetros. Finalmente, uma avaliação conjunta, considerando todas as classificações, demonstrou diferenças estatisticamente significativas nos parâmetros “Bordos” e “Tipo de Tecido Necrótico”, a favor do produto Bepanthene®.

Em suma, considerando os resultados obtidos, podemos concluir que o Bepanthene® apresentou melhor índice de cicatrização, em comparação com o Cyclopray®, podendo assim ser equacionado como uma alternativa à utilização de spray com antibiótico.

Palavras-chave: Bepanthene®; Cyclopray®; Descorna; Resistência; Antibióticos.

## **Abstract**

### **Comparative study of the healing process of disbudding wounds using Bepanthere® or Cyclopray®**

The process of disbudding/dehorning is common in most dairy farms, benefiting the handling, transportation and welfare of the animals. The most prevalent method is cauterization, responsible for destroying the horn bud and horn generating tissue. After the procedure, usually, the wounds are treated with an antibiotic based spray. Nowadays, antibiotic resistances are associated with high rates of morbidity and mortality, both in human and veterinary medicine, highlighting the need to invest in the monitoring of antibiotic use, new policies and the development of alternative treatment, in favor of One Health. The goal of this prospective study is to minimize the use of antibiotics in production animals, resorting to alternative treatments of disbudding wounds. The Bepanthere® (Dexpanthenol) is a plausible option, since it is widely used in human medicine for the treatment of skin irritations and burns.

The comparison of the healing process of disbudding wounds, treated with Bepanthere® or Cyclopray® (Chlortetracycline), was achieved through the presentation of images of the lesions to a panel of blind evaluators, constituted by seven veterinarians, five veterinary medicine students, and five human medical field nurses. In order to classify the lesions, the panel applied an adapted format of a verified healing scale, Bates-Jensen Wound Assessment Tool, incorporating seven parameters of evaluation, culminating in a total value, used in the statistical analyses.

In the veterinarians' evaluation, there was a statistically significant difference (P value <0.05), between the two products, for the parameter "Edges", indicating that the Bepanthere® is superior to the Cyclopray® in this specific parameter of healing. The assessment of the veterinary students showed a statistically significant difference, between the products, for the parameters "Exudate Type" and "Exudate Amount", showing the superiority of the Bepanthere's® action in the mentioned parameters. The evaluation presented by the nurses from the human medical field showed no statistically significant difference between the two products for any of the parameters. Lastly, the joint assessment, considering all of the evaluations, demonstrated statistically significant differences, between the two products, for the parameters "Edges" and "Necrotic Tissue Type", in favor of the Bepanthere®.

These findings lead us to conclude that the Bepanthere® presented a better healing index, compared to the Cyclopray®, allowing it to be considered as a safe alternative to the antibiotic based spray.

Key-words: Bepanthere®; Cyclopray®; Disbudding; Dehorning; Resistance; Antibiotics.

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## **List of Abbreviations and Symbols**

® - Registered Trademark

ARGs – Antibiotic Resistance Genes

DGAV – Direção Geral da Alimentação e Veterinária

DNA – Deoxyribonucleic Acid

GCs – Glucocorticoids

HGT – Horizontal Gene Transfer

MMPs – Matrix Metalloproteases

NA – Non-applicable

NSAIDs – Non-steroidal Anti-inflammatory Drugs

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## **I. Internship report**

In order to complete the Integrated Master's Degree in Veterinary Medicine, from the University of Lisbon, I completed the final internship in the span of six months, divided between two places. The first portion of my internship had a duration of three months, from the 14<sup>th</sup> of September to the 6<sup>th</sup> of December 2021, under the guidance of Professor Dr. George Stilwell. The second and final part of my internship, from the 10<sup>th</sup> of January to the 28<sup>th</sup> of March 2022, was spent in Centro Hospitalar Veterinário Limiavet, in Ponte de Lima.

During the first part of the internship, I was accompanied by another colleague, Luar Nell, and we participated in several field trips from "Clínica de Espécies Pecuárias", with the fifth year students. During these outings, we had the opportunity to visit an extensive number of farms, each with different protocols and procedures:

- Fonte Leite – Exploração Agrícola e Pecuária, S.A.;
- Barão & Barão, Lda;
- JMPC Lda;
- Pecbom – Produção de Leite, Lda;
- Agriangus Unip Lda;
- De Levie – Agriculture Produce Portugal, Lda;
- Casal de Quintanelas Lda.

In this time frame, I also gathered the necessary data for the experimental part of this master's thesis, resorting to animals from a Portuguese farm to develop a comparative study of the healing process of disbudding wounds, using Bepanthene® or Cyclospray®. The study consisted on doing the normal procedure of disbudding, following the regular protocol of the farm, and applying the two products to each animal, Bepanthene® on the right side and Cyclospray® on the left. Posteriorly, the wounds were photographed for the evaluation of the lesions, using a preexisting assessment tool, in order to determine the superior product for the healing of this type of wounds. The process of disbudding and the data collection were supervised by Professor Dr. George Stilwell and, on occasion, by Professor Dr. Ricardo Bexiga.

The second portion of my internship was spent at Limiavet, a veterinary hospital with excellent facilities and integrating a multifaceted team, constituted by six veterinarians and six veterinary nurses. The hospital is composed of a reception area for the patients, with commodities for the various animals, two consultation rooms and a laboratory, equipped with the different tools needed for an examination and diagnostics, a library with extensive literature, and three rooms dedicated to the hospitalization of animals, allowing for the separation of cats, dogs and, most importantly, animals with infectious diseases. Limiavet is also equipped with X-ray and ultrasonographic machines, valuable diagnostic tools, and a

room dedicated to small animal surgery. The hospital is also equipped with multiple vans filled with all the necessary material for outpatient consultations.



**Figure 1: Centro Hospitalar Veterinário Limiavet facilities**

Legend: Surgical room (top left corner), hospitalization area (top right corner), consultation room (bottom left corner) and the laboratory (bottom right corner).

During the internship, I had the opportunity, along with my fellow interns, of attending and successfully completing a course regarding the “Welfare Quality® protocol for fattening cattle (on-farm)”, lectured by Professor Dr. George Stilwell and evaluated by Professor Dr. Christoph Winckler.

For the duration of my internship, I was able to participate in various activities related to the small animal practice, such as:

- Discussion of clinical cases;
- Interpretation of laboratory results;
- Execution of physical exams;
- Preparation and administration of various medicines;
- Vaccinations and deworming's;

- Elaboration of vaccination and deworming plans;
- Collection of various different types of biological samples;
- Execution of x-rays and ultrasounds;
- Clean and bandage wounds;
- Participation in different surgeries, including monitoring of general anesthesia;
- Natural and caesarean births;
- Euthanasia.

Considering the portion of the internship dedicated to production animals, many activities were developed during this time:

- Discussion of clinical cases;
- Interpretation of laboratory results;
- Execution of physical exams and investigation of decreases in milk production;
- Preparation and administration of various medications;
- Vaccinations and deworming's;
- Collection of various different types of biological samples;
- Participation in different surgeries;
- Outpatient consultations and treatments;
- Pregnancy diagnosis and other modalities related to reproduction;
- Natural and caesarean births;
- Hoof trimming;
- Evaluation of production parameters;
- Euthanasia.

## II. Literature review

### Introduction

The prevention of horn growth (disbudding) or horn removal (dehorning) are common practices in many dairy farms, since animals subjected to these procedures tend to be easier to manage, facilitate the process of transport, reduce substantially the occurrence of injuries and contribute to an improvement of the welfare, not only of the animals but also of the farm workers (Stafford and Mellor 2005). The adequate age to perform this procedure varies concerning the breed of the calves, since dairy calves are, usually, disbudded in the first weeks following birth, at four to eight weeks (Stilwell et al. 2010), while beef breeds only undergo the process of disbudding once they are weaned, considering they are much less manipulated in comparison to dairy calves (Stafford and Mellor 2011).

The disbudding process can be accomplished through different methods, but the most common is the cauterization disbudding, using a heated iron to destroy the horn bud and horn generating tissue (Sutherland et al. 2019). Following the intervention, usually, an antibiotic based spray is used on the wounds resulting from the procedure, in order to avoid the development of infections.

Nowadays, antibiotic resistances have become a worldwide challenge, since they're associated with high rates of morbidity and mortality (Akova 2016). The difficulty in mitigating antibiotic resistance lies in the improper use of antibiotics and lack of information concerning this important topic (Frieri et al. 2017). Broad spectrum antibiotics continue to be recklessly used in unnecessary situations, contributing to this growing problem, especially in a time when new treatments for bacterial infections are limited (Akova 2016). In this sense, there is a need to invest in the monitoring of antibiotics, new policies regulating their use and new treatments alternative to the use of antibiotics (Velez and Sloan 2016).

Considering the presented information, the idea for this study emerged, with the main objective of minimizing the use of antibiotics in production animals, considering alternative treatments for the wounds resulting from the process of disbudding. The Bepanthene® was the product considered as the substitute of the antibiotic based spray, Cyclopray®. The Bepanthene® is heavily used in human medicine in the treatment of burns and skin irritations, promoting cellular division and migration, lipid production, tissue growth and reducing inflammation (Bayer 2021).

## **1. Animal welfare and the need to practice disbudding/dehorning.**

The Welfare Quality® Assessment protocol (2009, p.14) for cattle defines animal welfare as “a multidimensional concept” that “compromises both physical and mental health and includes several aspects”. It’s not possible to establish a specific metric for the measurement of animal welfare, and the various aspects considered for the evaluation have distinct meanings for each person that interprets their essence (Welfare Quality 2009). The protocol establishes four major principles in the assessment of welfare: good feeding, good housing, good health and appropriate behavior. For each of the principles, a set of welfare criteria must be analyzed, in order to guarantee the well-being of the animals. In the case of our study, the most important criteria is associated with the “good health” principle, elevating the importance of the absence of injuries, absence of disease and, lastly, absence of pain induced by management procedures, such as disbudding and dehorning (Welfare Quality 2009).

When discussing animal welfare, it is important to consider the “five freedoms”, stipulated by the “Farm Animal Welfare Council”, which are: freedom from hunger and thirst; freedom from discomfort; freedom from pain, injury and disease; freedom to express normal and natural behavior; and freedom from fear and distress (DGAV 2018). Beyond these basic guidelines, in Portugal there is legislation in place, designed to protect and safeguard production animals: Decree-law n.º 64/2000 of April 22 and Decree-law n.º 48/2001 of February 10.

Nowadays, certain surgical and non-surgical procedures are required in dairy cattle farms, in order to facilitate the handling of the animals (DGAV 2018). Disbudding (“to remove the horn buds of (calves, lambs, and kids) to prevent horn growing” (HarperCollins Publishers 2022)) and dehorning (“to remove the horns” (HarperCollins Publishers 2022)) are included in the group of procedures capable of improving the welfare of animals and cattle handlers (Stafford and Mellor 2005; Gottardo et al. 2011). Hornless cattle are less prone to cause injuries, easier to manage/handle, require less space and are less likely to suffer injuries trying to fit their head through the feeding racks (Stookey and Goonewardene 1996). Horned animals tend to suffer from hide damage and bruising, especially during transport, which can result in economic losses (Meischke et al. 1974), and present a risk, not only for other animals, but also to veterinarians and farm workers during routine procedures, such as milking, hoof trimming and calving (Gottardo et al. 2011).

## **2. Anaesthesia and analgesia for disbudding/dehorning**

The process of horn removal can be completed without any type of analgesia or anaesthesia, provided that the animals are properly restrained, which is made easier with young animals. However, the biggest issue surrounding the dehorning and disbudding of cattle is the pain associated with the methods that are used, and the response of the animals to the pain that is inflicted (Stafford and Mellor 2011).

Disbudding with chemicals may seem ideal, since the application of the caustic paste does not invoke an immediate response from the animal, falsely implying that this procedure will not affect the welfare of the calves, short and long term (Stafford and Mellor 2011). However, a rise in plasma cortisol occurred in the hour following the application of the paste, only returning to normal levels four to twenty-four hours following the procedure (Morisse et al. 1995). In terms of behaviours related to pain, the calves that underwent chemical disbudding expressed head shaking and rubbing right after the application, but also other specific behaviours three to four hours following, like inert lying, decreased grooming and restlessness (Morisse et al. 1995; Stilwell et al. 2009). Studies show that the application of local anaesthesia, when in combination with non-steroidal anti-inflammatory drugs (NSAIDs), eliminated not only the cortisol responses, but also the development of pain related behaviours (Stilwell et al. 2009).

When disbudding is no longer possible, the dehorning of calves must be carried out by amputation, related with acute and prolonged pain (Stafford and Mellor 2011). The amputation causes a significant rise in cortisol levels, reaching a peak in the thirty minutes after the procedure, followed by a decrease to a plateau that lasts about five to six hours, before reaching the normal cortisol levels, nine hours after the procedure (Cooper et al. 1995; Petrie et al. 1996; Sylvester et al. 1998). In the hours following the amputation, calves presented characteristic behaviours correlated to pain, such as tail and head-shaking, ear-flicking, increase in the time laying down, and decrease in rumination, grazing and grooming (Mcmeekan et al. 1998; Sylvester et al. 1998). However, the anaesthesia and analgesia using NSAIDs used before dehorning eliminates the cortisol response and behaviours related to pain, alleviating the acute and inflammatory pain associated with the procedure (Mcmeekan et al. 1998; Stafford and Mellor 2011).

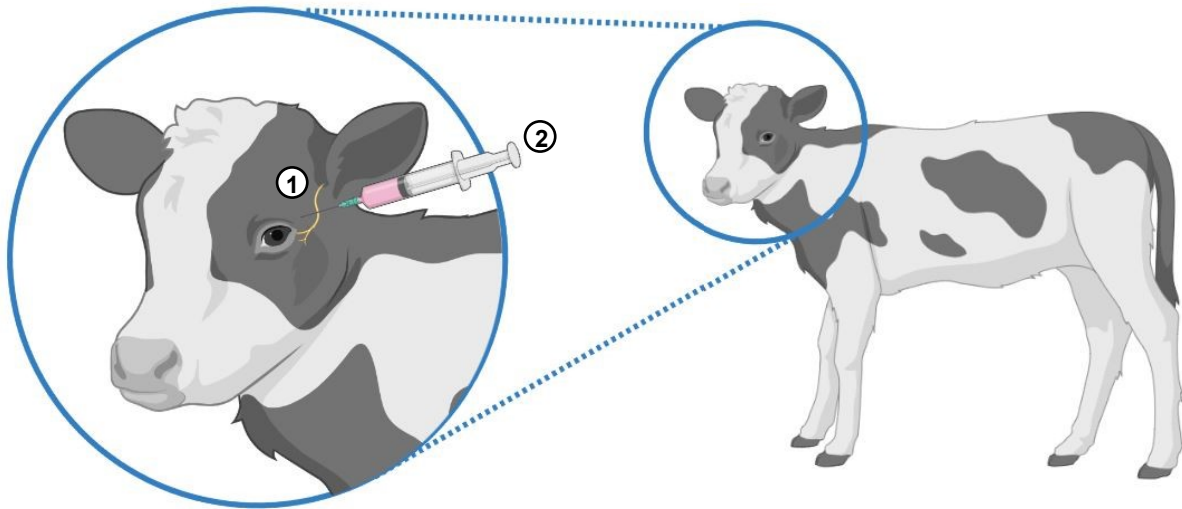
Cautery disbudding is the most used method to accomplish horn removal. During the procedure, the animals struggle, presenting characteristic behaviours like rearing, pushing, head jerking, moving and falling down, indicating pain related with the process (Graf and Senn 1999; Herskin and Nielsen 2018). Following horn removal, cortisol levels increase, reaching a peak thirty minutes following the procedure, returning to normal levels two hours after the removal (Petrie et al. 1996). Considering pain related behaviours, the calves presented an increase in head shaking and rubbing, grooming, hind-leg kicks, standing up or lying down

time, and a decrease in rumination (Morisse et al. 1995; Graf and Senn 1999). The administration of local anaesthesia abolishes the previously mentioned pain related behaviours displayed by calves during the procedure, and the addition of NSAIDs, like carprofen, alleviates the pain following disbudding (Milligan et al. 2004).

The sedation of the animals using xylazine facilitates the process of local anaesthesia, reducing the activity of the animal during the process, but it does not significantly influence the behavioural and pain responses presented by the animals (Stilwell et al. 2010).

Disbudding and dehorning, and the pain associated with the procedure, can be avoided by breeding hornless cattle (Stafford and Mellor 2011), and it is relatively easy to do so, since horns are an autosomal recessive trait, while polledness is a dominant trait (Long and Gregory 1978). According to research, polled and horned cattle of various breeds do not differ in weight gain, calf survival or fertility (Frisch et al. 1980; Stookey and Goonewardene 1996).

In order to progress with the process of disbudding, the importance of local anaesthesia has been proven. Local anaesthetics prevent the generation and propagation of action potentials, responsible for the nerve impulses, by binding directly with neuronal voltage-gated sodium channels (Yanagidate and Strichartz 2007). The usually performed cornual nerve block (figure 2) uses an 18- or 20-gauge needle, which is inserted lateral to the temporal ridge of the frontal bone and rostral to the horn base. After insertion, aspiration is performed in order to guarantee that the needle is inserted subcutaneously (Skarda 1986). Following negative aspiration, the needle is directed towards the horn, and five to ten millimetres of 2% lidocaine are infiltrated on the perineural space surrounding the cornual nerve, a “branch of the zygomaticotemporal portion of the ophthalmic division of the trigeminal nerve” (Stock et al. 2013, p.20). When cattle presents longer horns, there is a need to block the cutaneous branches of the second cervical nerve, accomplished with the injection of a local anaesthetic, caudal to the horn (Skarda 1986).



**Figure 2: Schematic representing the cornual block procedure (created with BioRender®)**

Legend: 1 – Cornual nerve (branch of the zygomaticotemporal portion of the ophthalmic division of the trigeminal nerve); 2 – Local anaesthetic.

### **3. Techniques and tools for disbudding/dehorning.**

Since the development of horns in some beef breeds occurs much later than in dairy breeds, and calves born in extensive herds, contrary to dairy calves, are rarely handled until they are five to six months old, it is difficult to determine an appropriate age to remove the horns (Stafford and Mellor 2011). Usually, dairy calves are disbudded in the few weeks following birth, when the horns are easily palpable, at four to eight weeks of age (Stafford and Mellor 2005; Stilwell et al. 2010). Opposite to what happens with dairy calves, beef breeds only undertake this procedure when they are weaned, at eight to ten months, demanding amputation of the horn due to its size (Weaver et al. 2005; Stafford and Mellor 2005).

Considering that dehorning and disbudding are common practices in most dairy farms, there has been a growing need to develop techniques and tools to perform these procedures in less time and in the most safe, efficient way (Stafford and Mellor 2011). The most common methods for disbudding/dehorning include cautery, caustic paste, or cutting off the horns, through the use of a saw, embryotomy wire, shears, or scoop (Sylvester et al. 1998). However, in the last couple of years, new methods for the disbudding of cattle have surfaced, namely the use of liquid nitrogen and clove oil. Unfortunately, these methods cannot be safely applied, since the nitrogen is ineffective, and although the clove oil showed promising results, there is not enough evidence regarding tissue damage and pain associated with the application (Sutherland et al. 2019).

### 3.1 Chemical disbudding

Chemical disbudding is a common form of horn removal in which a paste or a stick of sodium hydroxide or potassium hydroxide is applied to the horn, illustrated in figure 3 (Weaver et al. 2005), burning the tissues as long as the chemical is present, culminating with the destruction of the horn bud. The chemical method of disbudding is considered time consuming, since the area surrounding the horn must be scarified and defatted prior to the application of the paste, and demands various re-applications of the product, to ensure the efficiency of the process (Stafford and Mellor 2005). This method is also not very safe, since the chemical may spread to the neighbouring tissues, causing unnecessary injuries to the animal (trauma to the eye or the surrounding skin is common). Other animals may be affected by licking the product or cause damage to the udders of lactating cows (Stafford and Mellor 2011).



Figure 3: Chemical disbudding using caustic paste (Bauer 2015; Naylor 2015)

### 3.2 Dehorning by amputation

Disbudding occurs when horn buds are around five to ten millimetres long, allowing their removal using a disbudding iron or caustic paste (Stafford and Mellor 2005). However, once the horns grow longer than ten millimetres, attachment to the underlying frontal sinus occurs, requiring amputation of the horns, as shown in figure 4 (Stock et al. 2013). This method of dehorning has various techniques, accomplished with the use of different tools, such as a saw, scoop, guillotine shears or embryotomy wire (Sylvester et al. 1998).



**Figure 4: Technique of dehorning by amputation, using an embryotomy wire (original image)**

### **3.3 Cautery disbudding**

The cautery method of horn removal (figure 5) involves searing a heated iron, usually with a concave tip, into the horn bud and its surrounding tissue, for approximately fifteen seconds, in order to destroy the horn bud and the horn generating tissue (Weaver et al. 2005; Sutherland et al. 2019). The procedure results in two circular wounds, involving the epidermis and dermis (Adcock and Tucker 2018). The bar can be heated with electricity or gas, and this procedure should be performed on calves within the first four to six weeks after birth, when the horns are easily palpable, with five to ten millimetres of length (Stafford and Mellor 2011).



**Figure 5: Cautery disbudding, using a heated cautery (original image)**

#### **4. Disbudding/dehorning legislation**

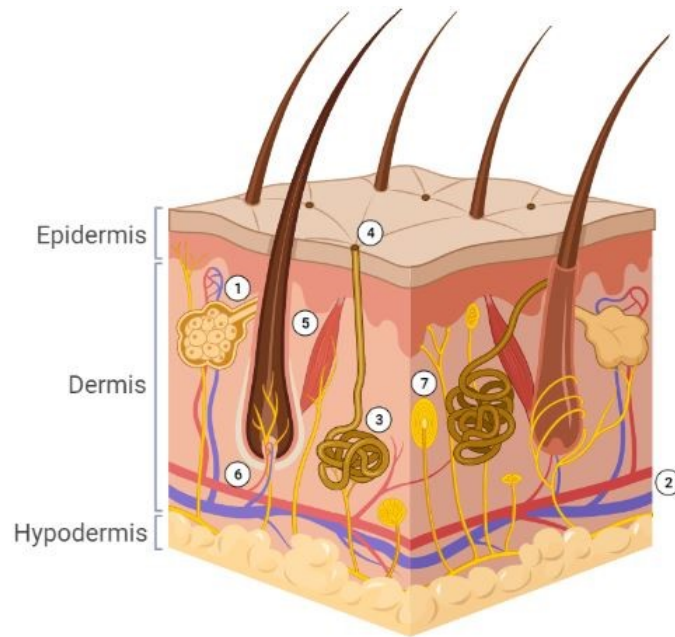
Dehorning and disbudding are regulated differently in different countries. In Portugal, Direção Geral da Alimentação e Veterinária (DGAV) determines that the procedure of disbudding must take place as soon as possible, before the calf turns two months old, when the horn bud starts to be visible and ahead of its fusion with the skull (DGAV 2018). The procedure must be executed by a veterinarian or by an experienced person, under the close supervision of a veterinarian. The removal of the horns should be carried out only if it benefits the welfare of the animals, using a cautery, always under local anaesthesia and analgesia. Disbudding by chemical cauterization is heavily advised against, since it does not meet the standards for the welfare of the animals (DGAV 2018).

As a precaution, the procedure should be performed in spring or fall, to avoid the presence of flies and other insects that may interfere with the process of wound healing. After the removal, the wound must be protected in order to avoid external contamination (seeds, grass, straw, bedding or silage). The straw intended to feed the animals should be placed strategically, at a level that reduces the risk of it falling on the animals head, avoiding another source of contamination (DGAV 2018).

DGAV also lists a number of indicators that should be assessed in order to determine the animal's welfare, such as mortality rate, behaviour, physical appearance, rate of complications after the procedure and changes in weight/body condition (DGAV 2018).

#### **5. Structure and function of the skin in cattle**

The skin, as presented in figure 6, is constituted of two layers, the epidermis and dermis, and under the skin it is possible to identify the hypodermis (Tobias and Johnston 2012). The outer layer, the epidermis, acts as a protective barrier, being thinner in parts of the body where hair coverage is denser, and thicker in places with exposed skin (Fossum et al. 2019). As an avascular layer, the epidermis receives nutrients from the fluid that penetrates deeper layers and from dermal capillaries. The dermis is thicker, composed of collagenous, reticular, and elastic fibres encapsulated in a substance constituted of mucopolysaccharides. Furthermore, this layer contains fibroblasts, macrophages, plasma and sebaceous cells, but also blood and lymphatic vessels, nerves, hair follicles, glands, ducts and smooth muscle fibres (Fossum et al. 2019). Lastly, underneath both of these layers, there is the hypodermis, also called subcutaneous tissue, containing adipose and loose connective tissue (Tobias and Johnston 2012).



**Figure 6: Schematic of the skin layers (created with BioRender®)**

Legend: 1- Sebaceous gland; 2 - vein (blue) and artery (red); 3 - sweat gland; 4 - sweat pore; 5 - muscle; 6 - hair follicle; 7 - nerve.

Concerning the vascular irrigation of the skin, it can be segregated into three divisions: the superficial or subpapillary plexus, the middle or cutaneous plexus and the deep, subdermal or subcutaneous plexus (Tobias and Johnston 2012). The subpapillary plexus is located on the outer layer of the dermis, with vessels projecting to the epidermis in order to irrigate the area. The cutaneous plexus irrigates the sebaceous glands and strengthens the vascularization of the hair follicles, tubular gland ducts and arrector pili muscles. Lastly, the subdermal plexus, vital in order to guarantee the viability of the skin, irrigates the hair follicles, tubular glands, arrector pili muscles and the deeper portion of the gland ducts (Fossum et al. 2019).

## 6. Types of wounds

The term wound refers to an injury involving the partition of tissues or rupture of the mucous membrane or integument. Typically, a skin injury is caused by an external source that disrupts the integrity of the skin (Yamaguchi and Yoshikawa 2001; Morton and Phillips 2016).

A wound can be defined as an open wound, when the whole thickness of the skin has been disconnected, including injuries like lacerations, avulsions and punctures; or a closed wound, when only a part of the layers that constitute the skin are involved, including abrasions, ulcers and thermal burns (Theoret and Schumacher 2016).

When it comes to infection, the wounds can be separated into different categories, from one to three, corresponding to a clean, contaminated or infected wound (Rizzo 2019). A class

one or clean wound (zero to six hours old) presents minimal contamination and tissue trauma, exhibiting insufficient organism replication to cause an infection. When referring to a clean wound, it's implied that the injury was produced under aseptic conditions (surgical incisions integrate this group), contrary to what happens with contaminated and infected wounds (Tobias and Johnston 2012; Rizzo 2019). A class two or contaminated wound (six to twelve hours old) shows a considerable replication of microorganisms ( $>10^5$  of bacteria per gram of tissue), but it may not have reached a critical level of multiplication. Lastly, a class three or infected wound (older than twelve hours) presents microorganism replication to a degree that may be consistent with the instalment of an infection, presenting a challenge in terms of healing and treatment (Tobias and Johnston 2012).

In order to treat a wound in the most adequate way there is a need to assess what type of wound we are dealing with.

### **6.1 Abrasion**

An abrasion is an epithelial injury that involves the superficial layers of the skin (Tobias and Johnston 2012; Theoret and Schumacher 2016), caused by blunt trauma or shearing forces, usually related to scarce bleeding and a rapid healing time. Although this type of wound is superficial, it is sensitive to pressure or to the touch (Fossum et al. 2019).

### **6.2 Penetrating or puncture wound**

An object that penetrates deep into the skin is responsible for causing a puncture wound, which can be defined as a deep skin opening associated with an ideal anaerobic environment that facilitates the growth of bacteria, causing tissue contamination and damage (Volk and Bohling 2013). The mass and velocity of the object responsible for the injury will influence not only the width and depth of the wound, but also the suffered tissue damage (Fossum et al. 2019).

### **6.3 Laceration**

The depth of the laceration varies between deep and superficial, commonly presenting irregular edges, usually with minimal trauma (Theoret and Schumacher 2016; Fossum et al. 2019). However, these types of wounds are usually associated with severe damage to the underlying tissues, like muscle and tendons, and are accompanied by bruising and considerable pain (Tobias and Johnston 2012; Theoret and Schumacher 2016).

## **6.4 Avulsion and degloving**

Avulsion wounds result from the tear of tissues from their anchor points, resulting in skin flaps (Fossum et al. 2019). When it comes to a degloving injury, associated with a broad loss of skin, deep tissue and, in some cases, bone (Volk and Bohling 2013), there's two types that must be taken into consideration: anatomic degloving and physiologic degloving. With anatomic degloving, the skin and other deep tissues are ripped from the limb, while in physiologic degloving the skin surface remains intact, but is completely disconnected from the neighbouring tissues and blood supply (Tobias and Johnston 2012), which may lead to necrosis of the skin.

## **6.5 Decubital ulcer**

Another type of wound can appear if the animal is laying down, for prolonged periods of time, with prominent parts of the bone in contact with hard surfaces, causing compression of the skin and underlying tissues, resulting in a decubital ulcer. In this type of injury, there's loss of skin and the damage may extend to the underlying soft tissues (Tobias and Johnston 2012).

## **6.6 Thermal burn**

Lastly, and the most relevant type of wound for this study, is the thermal burn, resulting from the contact of a source of heat with the skin (Tobias and Johnston 2012), ensuing various levels of injury to the tissue, highly prone to infection and sepsis. Most commonly, animal burn wounds result from accidental or malicious environmental exposure or they are iatrogenic (Volk and Bohling 2013), such as the burns resulting from the procedure of disbudding. Thermal burns can be classified depending on the tissues affected, ranging from superficial partial (epithelial), deep partial (epithelial and partial dermal) to full thickness (epithelial and dermal). Burns can have a significant impact on the organism, since they're associated with large fluid, protein and electrolyte loss (Tobias and Johnston 2012).

Although burn wounds undergo the same healing process as other injuries, there are certain differences that may influence their healing (Stilwell and Laven 2020). After a burn wound is created, there is an increase in the susceptibility to develop infections, related to the alterations of the immune status, which may result in sepsis and aggravation of the inflammation (Rowan et al. 2015). The hypermetabolism and inflammation, dependent of the extent of the wound, heavily influence the process of wound healing, delaying epithelialization. Burn wounds present a zone where protein denaturation, degradation and coagulation occurs, and a second zone characterized by areas of ischemia and hypoxia, both resulting in the necrosis of the tissue. The third zone is defined by the presence of hyperemia, receiving increased blood flow due to inflammatory vasodilation (Rowan et al. 2015).

## **7. Stages of the healing process**

Wound healing is a complex process responsible for restoring the normal anatomy and functionality of the injured tissue, resulting in a continuous epithelial surface, involving local cells, vascularization and extracellular matrix (Bertone 1989). Furthermore, the process requires a plethora of mediators, such as cytokines, growth factors and chemokines, in order to initiate and guide the process until its completion (Lux 2021). This process includes a number of stages that must be carried out in chronological order, initiating a new phase after the previous one is complete. However, wounds may exhibit various phases simultaneously, in different sections of the wound (Wilhelm et al. 2017; Wang et al. 2018).

Concerning wound closure, there are three types of healing to be considered: first, second and third intention healing. Primary closure, or first intention healing, is the most beneficial type of closure (Rizzo 2019), and it ensues right after the lesion has been inflicted (six to eight hours old) and before the formation of granulation tissue, considering the animals condition, cleanliness of the wound and minimal contamination and tissue trauma (Monnet 2008; Tobias and Johnston 2012; Fossum et al. 2019). In this type of healing, the edges of the wound bind together using different techniques and tools (for example, sutures, staples and tissue glue), allowing the appositional healing, resulting in closure of the wound with good hemostasis, without tension or dead space (Monnet 2008; Tobias and Johnston 2012; Rizzo 2019). Class one wounds are eligible for primary closure, but class two wounds only go through first intention healing if the contamination and tissue trauma is not significant and is manageable at closure (Tobias and Johnston 2012).

Second intention healing occurs if the wound is not readily closed after it is inflicted, due to the presence of tissue damage, severe infection, considerable tissue loss and/or devitalization of the skin edges, relying on the formation of granulation tissue, contraction and epithelization for the closure of the wound (Monnet 2008; Fossum et al. 2019). This type of closure results in larger scars, since the edges of the wound are not opposed (Rizzo 2019). It is necessary to perform proper debridement and cleansing of the wound, in order to guarantee proper closure. However, a good outcome is not guaranteed, since new epithelium is frail, wound contraction can be excessive and cause changes in function, and reepithelization may not be efficient, leaving granulation tissue exposed (Monnet 2008; Tobias and Johnston 2012).

Lastly, the secondary closure, or third intention healing, occurs three to five days following the injury, when the wound presents a layer of granulation tissue, and is achieved by appositional closure (Fossum et al. 2019). This type of closure is performed in wounds with severe contamination, necrosis and extensive tissue trauma, allowing for the management of the open wound over time (Monnet 2008; Tobias and Johnston 2012). The appositional closure is performed on top of the granulation tissue, following proper debridement and cleansing (Tobias and Johnston 2012).

The healing process of wounds is divided into three distinct phases: inflammatory phase, repair phase and maturation phase.

### **7.1 Inflammatory phase**

In a healthy wound, the usual timeframe for the inflammatory phase, including hemostasis and inflammation, is seventy-two hours, but a severe contamination of the wound can delay the process (Wang et al. 2018).

As a result of skin injury, hemostasis takes place, in order to start the healing process. The escape of intravascular fluid, but also the obstruction of lymphatic drainage, causes swelling, redness and heat, characteristic of inflammation (Li et al. 2007). The process of hemostasis begins right after the damage of blood vessels, with the objective of stopping the flow of blood (Rizzo 2019). This is achieved by the release of vasoactive compounds (histamine, serotonin, bradykinin and catecholamines), that will lead to a temporary vasoconstriction (five to ten minutes) to limit the hemorrhage, followed by vasodilation, that allows the leaking of fibrinogen and clotting agents into the injury (Hosgood 2006; Fossum et al. 2019). The coagulation mechanisms are triggered by the release of thromboplastin, promoting the aggregation and adhesion of platelets. The platelets release chemoattractants and growth factors that stimulate further platelet aggregation, resulting in the formation of a platelet plug that slows the hemorrhage (Cross and Mustoe 2003; Li et al. 2007; Fossum et al. 2019). Lastly, the fibrin, converted from fibrinogen, creates a mature blood clot that provides hemostasis, a barrier to infection and fluid loss, but also a matrix for cell attachment (Yamaguchi and Yoshikawa 2001; Werner and Grose 2003; Rizzo 2019)

The inflammatory phase is characterized by the triggering of inflammation, in response to the injury sustained. The platelets are the first cells to arrive at the site of injury, releasing growth factors that attract leucocytes and other cells into the wound, that will bind to the provisional matrix previously formed. The first leucocytes to reach the wound are neutrophils, shortly after the injury is sustained, followed by monocytes and lymphocytes, (Werner and Grose 2003; Li et al. 2007; Balsa and Culp 2015; Wang et al. 2018). Neutrophils possess integrin receptors that promote interaction with the matrix, allowing for the debridement of the wound, associated with the killing of microorganisms and elimination of injured cells and bacterial products, achieved by the release of enzymes and toxic oxygen products, and the phagocytosis of debris and microorganisms (Cross and Mustoe 2003; Hosgood 2006; Wang et al. 2018). Monocytes are able to turn into macrophages, a cell type essential for wound healing, proliferating in response to the presence of foreign materials or pathogenic microorganisms (Hosgood 2006). Macrophages are responsible for the phagocytosis of debris, microorganisms and apoptotic neutrophils, but they also stimulate wound healing by producing chemotactic and growth factors, responsible for tissue formation and remodel (Balsa and Culp

2015; Fossum et al. 2019). Macrophages are vital in the transition from the inflammatory phase to the repair phase, since they are responsible for the migration and proliferation of cells, and for the production and conservation of the matrix, that will originate the granulation tissue (Yamaguchi and Yoshikawa 2001; Li et al. 2007; Fossum et al. 2019).

## **7.2 Repair phase**

The repair phase, usually, starts three to five days following the injury, but the beginning of this process depends on the quantity of inflammatory cells in the wound. Nonetheless, the monocytes will continue to migrate to the wound site, turning into macrophages, responsible for stimulating DNA and fibroblast proliferation (Cross and Mustoe 2003; Schultz et al. 2003; Fossum et al. 2019). The repair phase includes a myriad of processes in order to establish wound closure, including the formation of granulation tissue, fibroplasia, angiogenesis, re-epithelialization and wound contraction (Hosgood 2006; Li et al. 2007).

The granulation tissue is responsible for the protection of the wound and for filling defects (Fossum et al. 2019). This type of tissue results from the transformation of the extracellular matrix, a process stimulated by the macrophages. The matrix allows the migration of the fibroblasts and epithelial cells, enabling the process of fibroplasia and angiogenesis (Yamaguchi and Yoshikawa 2001; Schultz et al. 2003). The appearance of the tissue alters through time, changing from a bright red appearance, due to higher vascularization, to a pale presentation (Lux 2021).

Fibroplasia lasts two to four weeks (Fossum et al. 2019) and is characterized by the migration, through the fibrin strands in the clot, and proliferation of fibroblasts in the wound site, in consequence to the action of macrophages, cytokines and extracellular matrix molecules (Cross and Mustoe 2003; Balsa and Culp 2015). The fibroblasts are tasked with not only the synthesis of important proteins (collagen, elastin and proteoglycans), that will integrate the fibrous tissue, but also the production of proteases, tasked with the removal of damaged proteins, allowing the deposition of the newly produced proteins (Werner and Grose 2003; Hosgood 2006; Fossum et al. 2019). As time progresses, the collagen content of the wound increases, with a rise of type I versus type III collagen, accompanied by a decrease in fibroblast count (Fossum et al. 2019). Gradually, through the work of the fibroblasts, the provisional matrix is completely replaced (Li et al. 2007).

Angiogenesis is the process of neovascularization, beginning with the migration of endothelial cells to the wound (Fossum et al. 2019). This process is stimulated by growth factors, liberated after the degradation of the matrix by proteinases, cytokines released by macrophages, low oxygen tension (resulting from tissue trauma) and lactic acid (Liekens et al. 2001; Werner and Grose 2003). However, basic fibroblast growth factor and vascular endothelial growth factor are the most important in this process, since they are specific to the

angiogenesis process (Stupack and Cheresh 2004; Fossum et al. 2019). The creation of blood vessels starts with columns of capillary endothelial cells that will migrate to the wound, forming new blood vessels that will supply the wound with nutrients and oxygen (Schultz et al. 2003; Fossum et al. 2019). In time, the vessels will regress and involute, altering the appearance of the granulation tissue (Lux 2021).

Re-epithelization, four to five days following the injury, reestablishes the barrier against external infection and internal fluid loss, involving the migration, proliferation and differentiation of epithelial cells (Fossum et al. 2019). The process differs with the type of wound present, beginning rapidly after the injury in partial-thickness wounds, with keratinocytes migrating from the wound margins and skin appendages (Hosgood 2006). However, when it comes to full-thickness wounds, the process will not start until a layer of granulation tissue is present, filling the wound (Sorg et al. 2017). In order to migrate, the keratinocytes suffer a myriad of transformations, such as changing their shape to flat and elongated, form lamellipodia projections, create actin filaments in the cytoplasm, and lose the attachments to the basement membrane and other epithelial cells (Li et al. 2007; Sorg et al. 2017). These transformations allow the keratinocytes to reach the wound centre, where proliferation will occur, assuring the supply needed for wound coverage (Hosgood 2006). The migrating cells, located under the scabs, start to produce collagenase, responsible for the dissolution of the base of the scab, promoting its removal (Fossum et al. 2019). In the beginning, the newly formed epithelium is only one layer thick, but it will thicken over time, with the possibility of regenerating hair follicles and sweat glands (Fossum et al. 2019).

The process of wound contraction occurs simultaneously to granulation and epithelialization, allowing a decrease in wound size (Fossum et al. 2019). Wound contraction involves myofibroblasts, resulting from the transformation of fibroblasts, causing a reorganization of collagen and contraction at the edges of the injury (Li et al. 2007; Sorg et al. 2017). The process halts when the edges of the wound meet, when there is extreme tension, in the presence of necrotic tissue, or when myofibroblasts are deficient (Schultz et al. 2003). If the process of contraction stops before the granulation tissue is fully covered, that task will be accomplished by epithelialization (Fossum et al. 2019; Lux 2021).

### **7.3 Maturation phase**

The maturation phase is characterized by adequate deposition of collagen, which occurs seventeen to twenty days after the injury is sustained. At the same time, the collagen and cellular content of the matrix start to dwindle, due to the apoptosis of endothelial cells and myofibroblasts (Desmouliere et al. 1995; Balsa and Culp 2015; Fossum et al. 2019). The strengthening of the newly formed collagen is achieved with the alteration of the fibers orientation and increased crosslinking, improving tensile wound strength (Hosgood 2006;

Balsa and Culp 2015). Overtime, type III collagen fibres decrease, while type I fibers develop, and collagen fibers with no functional orientation get degraded by proteolytic enzymes (matrix metalloproteinases), secreted by various components of the extracellular matrix, such as epithelial and endothelial cells, fibroblasts and macrophages (Chakraborti et al. 2003; Visse and Nagase 2003; Fossum et al. 2019). In the seven to fourteen days following injury, wound strength grows rapidly, due to the accelerated collagen deposition, but remains impossible to regain the previous tissue strength, only achieving a maximum of 70-80% of the original strength (Levenson et al. 1965; Orgill and Demling 1988; Fossum et al. 2019).

## **8. Factors liable to influence wound healing**

Wounds come in various forms and result from a myriad of causes. However, wound healing is affected by a number of factors that can be placed into different categories, considering different criteria. The process of healing can be influenced by both internal and external factors to the host (Fossum et al. 2019). When it comes to host factors, there is a division into two separate groups: the local factors affect, in a direct manner, the wound's characteristics, while the systemic factors, concerning the health and disease status of the individual, influence the ability of healing (Guo and DiPietro 2010).

### **8.1 Local factors that influence healing**

Oxygenation constitutes an important factor for wound healing (Hunt et al. 1969). When the wound is first formed, the microenvironment is hypoxic due to a decrease in oxygen levels, resulting from metabolically active cells with high rates of oxygen use and a disturbance in vascular support (Tandara and Mustoe 2004). Initially, oxygen depletion aids in the wound healing process, since it serves as a signal that prompts the start of the healing, inducing cytokine and growth factor production from macrophages, keratinocytes and fibroblasts, and stimulates angiogenesis (Knighton et al. 1983). However, as time progresses and the oxygenation is not restored, the process of healing is heavily compromised, since oxygen is extremely important for the metabolism of the cells, indispensable in the process of energy production. Besides preventing wound infection, proper oxygen levels also promote wound contraction, stimulate keratinocyte differentiation, migration and re-epithelialization, also increasing the proliferation of fibroblasts and collagen synthesis (Bishop 2008; Rodriguez et al. 2008). As time goes on, and fibrosis of the wound starts to occur, trapped leukocytes release lysosomal enzymes and proinflammatory mediators that are liable to intensify the problem and, ultimately, lead to the destruction of tissue (Stadelmann et al. 1998). Ultimately, the proper level of oxygenation in wounds is needed in order to maintain the process of wound healing long term (Guo and DiPietro 2010).

Following a tear in the skin, the organisms that populate its surface start to proliferate in the underlying tissue, triggering an infection (Bishop 2008). Infected wounds can be classified considering the replication status of the micro-organisms and the infection: contamination (present non-replicating organisms), colonization (present replicating organisms without displaying tissue damage), local infection/critical colonization (present replicating micro-organisms and the start of local tissue damage) and invasive infection (replication of organisms resulting in host injury) (Edwards and Harding 2004; Balsa and Culp 2015). The inflammation is a normal and desirable process, aiding in the removal of contaminating micro-organisms. However, an ineffective decontamination is associated with an extended inflammatory phase, characterized by a prolonged rise of pro-inflammatory cytokines, increased levels of matrix metalloproteases (MMPs) and a decrease in naturally occurring protease inhibitors, placing the wound in a chronic state with impaired healing (Edwards and Harding 2004; Menke et al. 2007). Another problem that arises with the infection of the wound is the formation of complex communities integrating the micro-organisms that colonize the lesion, creating biofilms, more resistant to disinfections and conventional antibiotic treatments (Davis et al. 2008).

The location of the wound constitutes an important factor concerning the process of healing, since motion and tension, inherent to certain parts of the body, heavily limit the capability of healing (Fossum et al. 2019). Motion, and subsequent tension, can lead to an increase in the amount of scar tissue, widening the resulting scar, and ischemia and necrosis of the surrounding skin, in severe cases (Bertone 1989; Balsa and Culp 2015). Wounds located in places with a higher count of microorganisms (perianal, oral or other locations) are more prone to develop an infection (Guo and DiPietro 2010).

## **8.2 Systemic factors that influence healing**

Age constitutes an important factor due to related changes and delays in the process of wound healing. Healthy older individuals show a temporal delay in healing, due to altered inflammatory response (delayed T-cell infiltration and reduced phagocytic capacity of the macrophages)(Swift et al. 2001), but the process itself is not impaired in terms of quality (Gosain and DiPietro 2004; Keylock et al. 2008). Aging of the individuals is correlated with a number of physiological effects, including an increase in platelets aggregation and secretion of inflammatory mediators, defective macrophage function, decreased secretion of growth factors, diminished wound strength and delayed infiltration of immune cells, re-epithelialization, angiogenesis and collagen deposition (Gosain and DiPietro 2004).

The stress factor compromises the healing process of wounds by deregulating the immune system (Boyapati and Wang 2007). In this situation, glucocorticoids (GCs) are increased, proinflammatory cytokines are reduced, and chemoattractants, indispensable for

the initial inflammatory phase, are severely diminished (Godbout and Glaser 2006; Boyapati and Wang 2007). The hormone cortisol, released in stress filled situations, acts as an anti-inflammatory agent and modulates immune responses crucial in the beginning of the healing process, constituting an important part in the delay of the wound healing (Godbout and Glaser 2006).

Wound healing can be critically influenced by the action of glucocorticoids, as previously mentioned, since they suppress the differentiation and proliferation of immune cells, increasing the risk of infection, regulate gene transcription, reduce the expression of cell adhesion molecules, decrease collagen synthesis, and result in incomplete formation of granulation tissue and poor wound contraction (Burns et al. 2003; Sternberg 2006; Franz et al. 2007). However, the topical application of corticosteroids produces different results, accelerating wound healing, reducing pain and exudate, and diminishing the formation of hypergranulation tissue (Hofman et al. 2007).

Besides corticosteroids, there are other medications that can influence the process of wound healing, like non-steroidal anti-inflammatory drugs and chemotherapeutic drugs. Some NSAIDs, in specific concentrations, have shown an anti-proliferative effect, leading to a decrease in the number of fibroblasts, reduced wound contraction, defective angiogenesis, and delayed epithelialization (Dong et al. 1993; Jones et al. 1999; Guo and DiPietro 2010). When it comes to chemotherapeutic drugs, most of them are designed to halt cellular metabolism, cell division, and angiogenesis, delay cell migration, decrease matrix formation, prevent the proliferation of fibroblasts, impair wound contraction and, lastly, weaken the immune system, interfering with the process of wound healing (Burns et al. 2003; Guo and DiPietro 2010).

Healing can be slowed by a number of metabolic diseases, such as hyperadrenocorticism, uremia and diabetes mellitus (Balsa and Culp 2015). Diabetes mellitus is heavily associated with hypoxia, decrease in inflammatory response and chemotaxis (Tobias and Johnston 2012), fibroblasts and epidermal cells dysfunction, defective angiogenesis and neovascularization, reduced host immune resistance and neuropathy (Guo and DiPietro 2010).

The correct nutritional balance and caloric intake represent an important factor in wound healing, since nutrient deficiencies and malnutrition can have an enormous impact in wound healing (Burns et al. 2003). Carbohydrates, protein, energy, fat, vitamins, and minerals all contribute to a flawless healing process (Williams and Barbul 2012). Considering animals nutrition, an excessive weight also represents an impairment to wound healing, since overweight individuals tend to not only present a higher risk of developing different diseases, but are also more prone to wound infections, dehiscence, hematomas, seromas, and skin ulcers (Guo and DiPietro 2010). The presence of excessive adipose tissue is related with hypoperfusion and ischemia, impairing wound healing and promoting infections due to a delay

in antibiotic action (Wilson and Clark 2004). However, cachectic patients also present impairments to the process of wound healing, since they present decreased fibroplasia and a higher risk of infection (Bertone 1989).

## **9. Antibiotic resistance**

In a time when new treatments for bacterial infections are few and far between, it becomes crucial to address the topic of antibiotic resistance and how to mitigate its impact on public health. Resistance to antibiotics is a very current problem that demands urgent attention from researchers, policymakers, and prescribers, in order to stop it from evolving (Frieri et al. 2017). Furthermore, the growing cases of antibiotic resistance represent, not only a significant human cost, but also a huge economical setback, since it places a worldwide pressure on the development of alternatives for this problem (Bush et al. 2011; Velez and Sloand 2016).

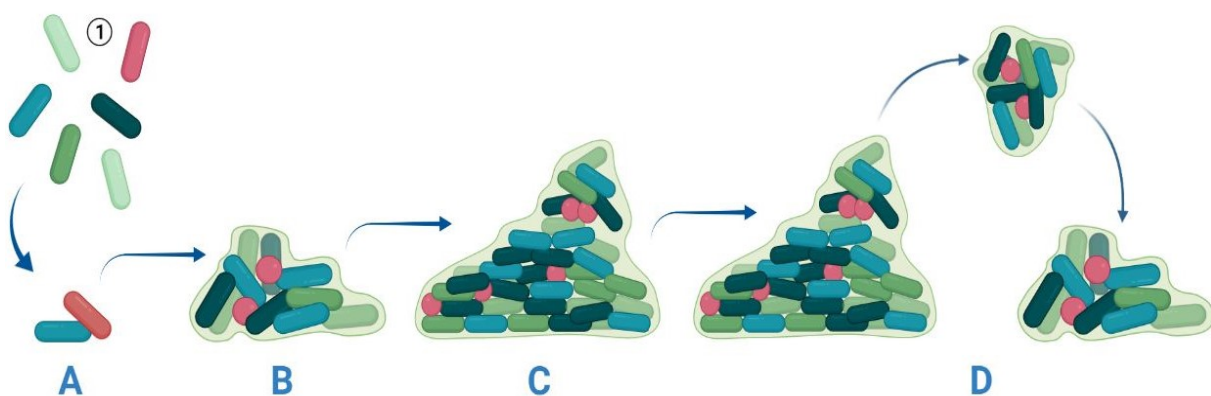
The high rate of mortality and morbidity (Akova 2016), associated with antimicrobial resistance, can be attributed to gram-positive and gram-negative bacteria being highly resistant to a wide variety of antibiotics, which results in infections that are very hard, if not impossible, to treat with conventional antibiotics (Chellat et al. 2016; Frieri et al. 2017). The escalation of this problem is mostly due to the overuse of broad spectrum antibiotics and the ease of dissemination, to various patients and the environment, of these resistant bacteria (Akova 2016). The fact that the world takes advantage of a global economy contributes to the problem at hand, facilitating the movement of goods and food that are liable to contribute to the dissemination of resistant bacteria (Bush et al. 2011).

Various antibiotic preparations, for human and animal use, often carry antibiotic resistance gene sequences, which are responsible for rapid increases in antibiotic resistance (Webb and Davies 1993; Bush et al. 2011). All types of bacteria (pathogenic, commensal and environmental) are capable of mobilizing bacteriophages and genetic material, developing a reservoir of antibiotic resistance genes (ARGs), called the resistome. A mechanism called horizontal gene transfer (HGT) enables antibiotic resistance to spread from commensal and environmental species to the pathogenic ones (Aminov and Mackie 2007; Von Wintersdorff et al. 2016). The resolution of this problem lies in the identification of the major reservoirs of resistance genes, present in humans, animals, and in the environment (Bush et al. 2011).

Biofilms are biological systems made of microbial cells that are associated in a functional community, and they can grow on various different surfaces and environments. An auto-generated biopolymeric matrix holds the microorganisms together, composed of polysaccharides, proteins, extracellular DNA and different elements from the host, such as immunoglobulins (Costerton et al. 2003). Disinfectant products, antibiotics and different components of the inflammatory system of the host are all ineffective against bacterial biofilms (Frieri et al. 2017).

Creating a biofilm, as shown in figure 7, begins with the reversible attachment of bacteria to a surface. However, at this time, the microorganisms are still susceptible to the action of antibiotics, only acquiring their resistance when they start binding irreversibly to the surface, multiplying and producing the polymer matrix around the microcolony, which is vital for the protection and balance of the biofilm (Høiby et al. 2011). Following biofilm formation, focal dissolution happens, releasing bacterial cells to different locations, enabling the formation of new biofilms (Webb et al. 2003).

Most of the time, a prolonged use of antibiotics is necessary to target and eliminate this type of bacterial cell formation; however, these approaches are usually in vain, perpetuating the infection and increasing antibiotic resistance (Ribeiro et al. 2016). As a means of avoiding and minimizing the concerning issues surrounding biofilms, a study of the pharmacokinetics and pharmacodynamics of antibiotics must be conducted, in order to establish an efficient course of treatment (Hengzhuang et al. 2014).



**Figure 7: Schematic of the formation of a bacterial biofilm (created with BioRender®)**

Legend: 1 – Bacteria;

A - Reversible attachment of bacteria to a surface; B – Irreversible binding to the surface and production of the biopolymer matrix around the microcolony; C – Multiplication of the bacteria and production of matrix; D – Release of bacterial cells to different locations, forming new biofilms.

The issue of antibiotic resistance can be addressed in both human and agricultural sectors through optimized surveillance, introduction of new and improved policies and by developing new treatment courses (Velez and Sloand 2016; Frieri et al. 2017). The development and use of new and improved technologies is imperative for the progress of the medical industry, allowing the detection of virulence and antibiotic resistance genes with techniques like whole-genome sequencing, favouring the emergence of new and revolutionary treatments (Bakour et al. 2016). The education of the general public constitutes an important measure, with the main objective of educating the population on the importance of bacteria

and the correct use of antibiotics, and how it contributes to their lives and well-being (Bush et al. 2011). Another imperative measure includes the elimination of prosperous conditions for the dissemination of bacteria, such as high population density, frivolous use of antibiotics, clean water shortages, and the inadequacy of the treatments for industrial and sewage effluents (Bush et al. 2011). Lastly, it becomes necessary to regulate the use of antibiotics, in order to stop the surfacing of new antibiotic resistant bacteria, allowing for the use of pre-existing working treatments, but also the development of new and/or repurposed antibiotics, as well as new approaches for dealing with infections (Velez and Sloand 2016; Frieri et al. 2017).

The spread of antibiotic resistance from the agricultural sector represents a long-lasting battle in the fight against resistant bacteria (Velez and Sloand 2016; Frieri et al. 2017). This problem stems, mostly, from the unruly use of antibiotics, but is also related with the recurrent use of these substances in order to promote the growth of animals, creating the perfect environment for the development of resistances (Velez and Sloand 2016). Considering this concerning problem, the development of strategies to prevent this type of situations becomes imperative, like establishing laws for the use of antibiotics, prohibiting their use as growth stimulators, avoid buying or the consumption of antibiotic fed meat, promote novel treatments, in alternative to antibiotics, and design farming subsidies that reward good practices, encouraging the correct use of antibiotics (Duckenfield 2013).

Studies have shown that the indiscriminate use of topical agents containing antibiotics is related to the development and spread of bacterial resistances, through the dissemination of resistance mechanisms across bacterial species (Williamson et al. 2017). Furthermore, the use of this substances topically is linked to reports of cross-resistance to systemic agents, potentiating the rise of new antibiotic resistances. In this sense, the development of alternative treatment approaches becomes imperative, in order to reduce the use of topical agents containing antibiotics and, consequently, the increase in bacterial resistance (Williamson et al. 2017). The use of topical antibiotics is also linked to a decrease in cellular function, delaying the process of wound healing (Stilwell and Laven 2020).

Considering the legislation of the use of antibiotics in veterinary medicine, the European Union established the Regulation (EU) 2019/6 of the European Parliament and of the Council of 11 December 2018 on veterinary medicinal products, and the Regulation (EU) 2019/4 of the European Parliament and of the Council of 11 December 2018 on the manufacture, placing on the market and use of medicated feed. These regulations control the application of antibiotics used to treat animals, avoiding the improper and abusive use of these substances, avoiding the rise of antibiotic resistances.

## 10. Bepanthere® as an alternative to Cyclopray®

Following the process of disbudding, it is common to apply a spray with antibiotic on the resulting wound, with the objective of preventing the development of infection. In this particular case, the spray that will be considered is the Cyclopray® 78.6 mg/g, for the “treatment and prevention of superficial skin infections caused by micro-organisms sensitive to chlortetracycline” (Virbac 2018). In terms of composition, the 200 ml spray contains 78.6 mg of Chlortetracycline HCl and 4,8 mg of one excipient that is used as a coloring agent, Patent blue V (DGAV 2021).

However, the growth of antibiotic resistances over the years requires the development of new strategies and treatments, in order to stop this concerning issue from growing beyond our control. In this sense, Bepanthere® was selected for this study, in order to determine if the product is able to replace the antibiotic spray in the treatment of disbudding wounds. Bepanthere® has been used in human medicine for several years, promoting the regeneration and healing of burns and irritations, by strengthening the natural skin barrier (Bayer 2021). The Bepanthere® contains 50 mg/g of the active substance dexpanthenol, also known as provitamin B5 essential for the regeneration of the skin, but also purified water, lanolin, liquid paraffin, white soft paraffin, protegin X, almond oil, beeswax, cetyl alcohol and stearyl alcohol (Bayer 2021).

When applied topically, the dexpanthenol acts as a moisturizer. Numerous studies have demonstrated that dexpanthenol is responsible for the stimulation of important genes involved in wound healing, promoting a rapid re-epithelialization and restoration of the skin barrier, and is also responsible for the increase in lipid production, essential for a rapid healing (Gorski et al. 2020). The compound is also linked to anti-inflammatory effects, when applied topically (Ebner et al. 2002), and is responsible for promoting fibroblast proliferation, both *in vitro* and *in vivo* (Abdel-Hamid et al. 1984; Ebner et al. 2002). Several *in vitro* studies, on human fibroblasts, exhibited an enhancement on cell proliferation and migration, attachment of fibroblasts and in the production of collagen (Pugliese et al. 1995).

Previous studies have shown the effectiveness of alternative treatments in the healing of disbudding wounds, such as the use of aluminium-based aerosol bandage (Huebner et al. 2017) and the use of a formulation containing topical anaesthetic and antiseptic (Stilwell and Laven 2020). These new approaches present viable alternatives for the use of antibiotic based spray, reducing the use of antibiotics in production animals. Concerning the economical side, the Cyclopray® presents a similar price when compared to the Bepanthere®, which means the replacement will not negatively impact the farms.

### **III. Experimental**

#### **1. Objectives**

Preventing the growth of horns, or posterior removal, are common practices among many dairy farms, since this procedure facilitates the handling of the animals, transport and contributes to the improvement of the general welfare. The process of disbudding can be carried out with various techniques, but cautery disbudding remains the most common, using a heated iron to eliminate the horn bud and the horn generating tissue. After the intervention, usually, an antibiotic based spray is applied in order to prevent the development of infection involving the wounds that result from the procedure.

Nowadays, antibiotic resistance poses a major threat, since it is associated with high rates of morbidity and mortality on a worldwide level. The difficulty in mitigating this resistances is mostly due to improper use of antibiotics and lack of information concerning the topic.

With this problem in mind, the idea for this study started to take shape, in an effort to reduce the use of antibiotics in production animals. As an alternative, Bepanthere® (Dexpanthenol) was chosen as a possible substitute to the antibiotic based spray, in this case being Cyclopray® (Chlortetracycline). The Bepanthere® is vastly used in human medicine to treat burns and irritations, promoting cellular division and migration, lipid production, tissue growth, reduces inflammation and increases the retention of fluids in the skin.

The main objective of this dissertation is to determine if the Bepanthere® is a suitable substitute to the Cyclopray® in the healing of disbudding wounds, through the comparison of the healing process for the two products, using a validated healing scale applied by a blind panel constituted by veterinarians, veterinary students and nurses from the human medical field.

#### **2. Materials and methods**

##### **2.1 Sample population**

All procedures were carried out with the approval of the farm and of the Ethics Committee (Comissão de Ética para a Investigação e Ensino – CEIE) from the Faculty of Veterinary Medicine, University of Lisbon. The committee recognized the safeguard of all ethical principles, approving the execution of the experimental protocol. In this study, all the animals used were from the same Portuguese farm. In this particular exploration, the calves are kept in individual boxes, lined with hay and with communication between them, until they are approximately three weeks old, and after that are transitioned to bigger pens, with other older calves, composing various age groups. Each box presents a metal support for the water and milk buckets, and a plastic bowl for the deposition of calf-starter. The bigger pens contain about ten to twenty animals, and include feeding stations and proper bedding for the animals.

There was no particular criteria considered for the selection of the animals, since the calves used for the study were the ones selected by the farm to undergo the procedure. The animals were, approximately, three weeks old, all females and belonging to the Holstein-Friesian breed.

A total of twenty-seven animals were evaluated for this study, divided into two groups based on when the procedure was completed. The animals that make up the two groups and the respective day of the disbudding are discriminated in table 1:

**Table 1: Identification of the animals that constitute the two groups and respective day of disbudding**

<b>Groups</b>	<b>Identification</b>	<b>Day of disbudding</b>
<b>Group 1</b> (13 animals)	3877; 3878; 3879; 3880; 3881; 3883; 3884; 3885; 3886; 3887; 3888; 3889; 3890	20 <sup>th</sup> of October, 2021
<b>Group 2</b> (14 animals)	3932; 3933; 3934; 3935; 3936; 3937; 3938; 3939; 3941; 3942; 3943; 3944; 3945; 3946	17 <sup>th</sup> of November, 2021

## **2.2 Methodology of disbudding**

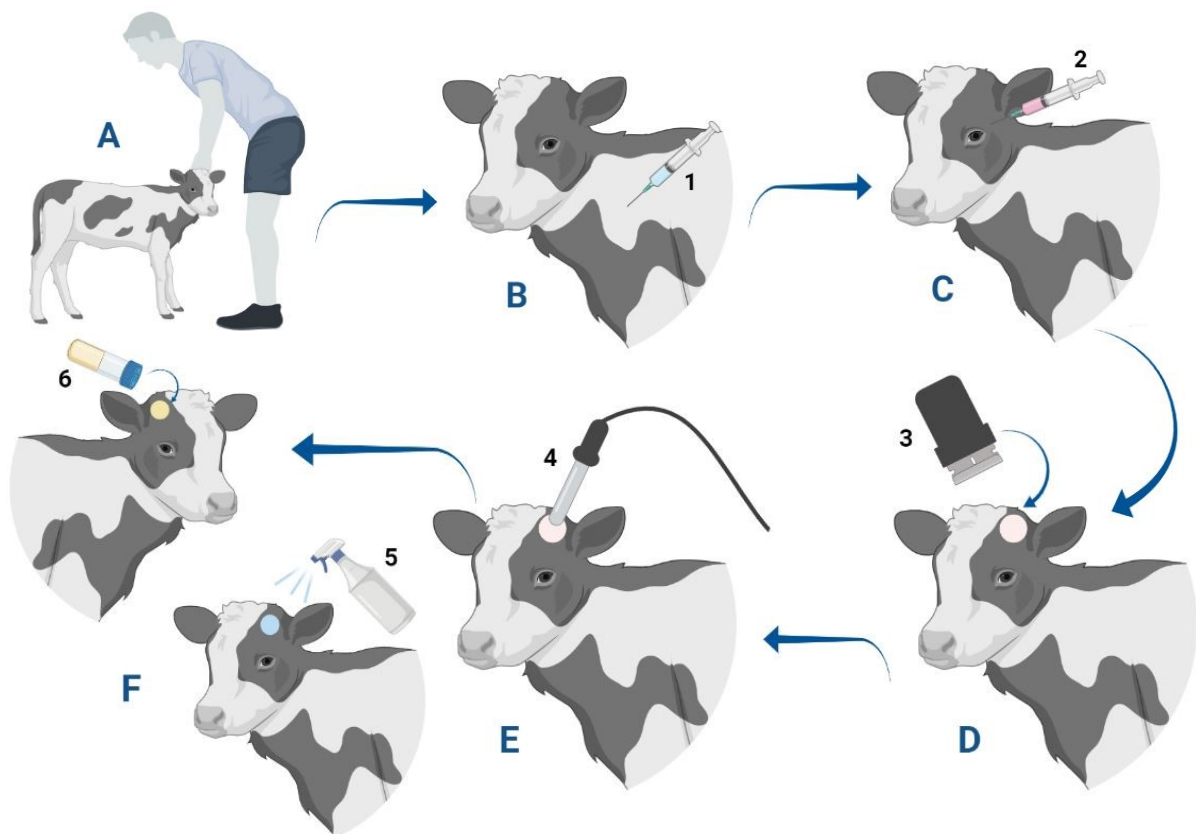
The process of disbudding, illustrated in figure 8, starts with the proper restraining of the animals, in order to facilitate the procedure and avoid accidents that may result in injuries to the animals as well as people involved in the process. In this particular farm, the calves are kept in individual boxes that contribute to the disbudding process, favoring the restraining of the animals, since it's a much smaller space that allows a better handling of the animal.

After the animal is properly restrained, anesthesia and analgesia were performed in advance, to guarantee the calf is under the effect of the drugs during the procedure. Procaine (Procamidor® 20 mg/ml) was used as local anesthetic. The cornual nerve block is achieved by using a 20-gauge needle that is inserted lateral to the palpable temporal ridge of the frontal bone, rostral to the base of the horn. The needle is pointed towards the contralateral eye and, after aspiration to guarantee the needle is subcutaneous, 6 ml of procamidor is injected (figure 9). Regarding analgesia, each animal received 1.5 ml of Carprofen (Carprosan® 50 mg/ml) subcutaneous prior to the procedure.

Performing trichotomy beforehand improves the asepsis of the procedure and facilitates the identification of the growing horn, which is essential before the process of disbudding begins. To perform the trichotomy, standard clippers were used, starting by

identifying the horn and cutting the hair around it, leaving a margin superior to two centimeters from the horn bud.

The process of disbudding starts roughly ten minutes after the administration of the drugs, using a cautery to remove the horn bud, as illustrated in figure 8 and 9. The procedure starts when the hot iron is applied against the skin of the animal, ensuring that the opening of the cautery aligns with the horn bud. After confirming the iron is placed in the right place, it's necessary to apply a downwards force, against the skull of the animal, and start a rotating motion, with wide movements, in order to remove the horn bud. As soon as the skin detaches from the underlying tissue, the iron must be placed diagonally and the direction of the force applied must be away from the animal's face, in order to cut the base of the horn bud. This process culminates with two circular burn wounds, on each side of the skull, that were treated with two different products: Bepanthene®, an amount roughly the size of a hazelnut, was applied to the wound on the right side of the skull, while Cyclospray® was used to treat the wound on the left side, as shown in figure 8 and 10.



**Figure 8: Schematic of the process of disbudding (created with BioRender®)**

Legend: A - Immobilization of the animal; B - Analgesia using 1.5 ml subcutaneous of Carprosan® 50 mg/ml (1); C - Local anesthesia with 6 ml of Procamidor® 20 mg/ml (2) on each side; D - Trichotomy with standard clippers (3); E - Disbudding with heated cautery (4); F - Application of Cyclospray (5) on the left wound and Bepanthene (6) on the right wound.



**Figure 9: Phases of the process of disbudding (original images)**

Legend: A - Local anesthesia with 6 ml of Procamidol® 20 mg/ml on each side; B - Disbudding with heated cautery.



**Figure 10: Burn wounds resulting from disbudding, treated with Bepanthen® on the right side and Cyclopray® on the left side**

Legend: Bepanthen® on the right side of the animal, left in the image; Cyclopray® on the left side of the animal, right in the image.

### 2.3 Assessment of the disbudding wounds

In order to evaluate the differences between the healing process of the wounds treated with Bepanthere® and Cyclopray®, periodic assessments were made to collect data and photographic evidence necessary for the evaluation. As stated previously, group one was disbudded in the 20<sup>th</sup> of October, while group two underwent the procedure on the 17<sup>th</sup> of November. Posteriorly, periodic assessments were made to both groups, making sure to establish strict time intervals for the evaluations, in order to guarantee a certain consistency to the results obtained. The dates of the assessments are listed in table 2:

**Table 2: Time intervals for the assessments of each group since the day of disbudding**

<b>Groups</b>	<b>Day of assessment</b>	<b>Days from disbudding</b>
<b>Group 1</b> (13 animals)	22 <sup>nd</sup> of October, 2021	Two days
	25 <sup>th</sup> of October, 2021	Five days
	9 <sup>th</sup> of November, 2021	Twenty days
	19 <sup>th</sup> of November, 2021	Thirty days
<b>Group 2</b> (14 animals)	19 <sup>th</sup> of November, 2021	Two days
	22 <sup>nd</sup> of November, 2021	Five days
	7 <sup>th</sup> of December, 2021	Twenty days
	17 <sup>th</sup> of December, 2021	Thirty days

Each group had four dates for the evaluation of the wounds and to collect photographic evidence of each of the lesions, used for a comparative assessment of the healing process of disbudding wounds using Bepanthere® or Cyclopray®. Subsequently, the pictures obtained were used to make a presentation where they were displayed randomly (description below) and given a specific code, in order to avoid a biased classification. In other words, each animal from the study (27 animals in total) had two pictures taken for each periodical evaluation, making up a total of eight pictures per animal used on the presentation, summing up to two hundred and sixteen (216) pictures to evaluate.

The evaluation of the healing process of each wound was made with the assistance of a validated healing scale, Bates-Jensen Wound Assessment Tool (Bates-Jensen 2001). This scale is constituted of thirteen parameters that must be evaluated on a scale of one to five, one being the best score and five being the worst. There was also the possibility of rating with “NA” for “Non applicable”, for dubious situations when the classifier does not know what to score or considers the parameter of classification does not apply. When the wound is rated for all the parameters, the total score is determined by adding all of the scores given throughout the evaluation. The higher the total score, the more severe the wound status. However, for this

particular study, the scale was modified in order to fit the requirements of the trial, since many parameters require measurements and other assessments that can't be completed by observing images. Therefore, the adapted Bates-Jensen Wound Assessment Tool only has seven parameters, capable of being evaluated through pictures: edges, necrotic tissue type, exudate type, exudate amount, skin color surrounding wound, granulation tissue and epithelialization (Attachment 1).

In an effort to obtain a representative evaluation of the lesions, a panel of evaluators/classifiers was created, composed of seven veterinarians, five veterinary medicine students and five nurses from the human medical field. The presentation containing the various coded wounds, the adapted Bates-Jensen Wound Assessment Tool and an excel sheet, to register the score for each parameter, were sent to each of the evaluators, providing all of the tools necessary to complete de classification.

## **2.4 Statistical Analysis**

The statistical analysis was conducted using the Microsoft Excel® 2013 (Seattle, WA, USA), for the organization of data, and the GraphPad Prism 9 software (La Jolla, USA), specifically for the design of graphics and statistical analysis. Before conducting any tests, for all continuous variables an assumption of normality was evaluated by the Shapiro-Wilks and Kolmogorov-Smirnov statistical tests, taking into consideration the respective measures of skewness and kurtosis, and visualizing histogram normal distributions.

If the normality tests show that one, or both of the variables, do not fit the assumption of normality ( $P > 0.05$ ), a nonparametric statistical test was conducted, namely the Wilcoxon matched-pairs signed rank test. This specific test is used to compare two paired groups, analyzing the differences between them, trying to establish if they are statistically significantly different from each other.

In order to determine if the difference between Bepanthe® and Cyclopray® was statistically significant ( $P < 0.05$ ), a Paired t-test was used, trying to evaluate the difference between two variables for the same subject, since each animal was treated with both products. For the Paired t-test a two-tailed P value was used, in which the area of distribution is two-sided, in order to test for the possibility of a relationship in both directions.

The distribution of the sample values, for each of the treatments and individual parameters, was assessed using a Two-way ANOVA, allowing the appraisal of similarities or inconsistencies between the evaluations of the classifiers.

Lastly, the ROUT method of identifying outliers was applied, used to detect any number of outliers among any sample. The test was conducted to determine if there was an observation that lies an abnormal distance from the other values in a sample. The presence of outliers increases the error variance and reduces the power of statistical tests.

For the analyses, it was necessary to start by considering the classifiers (veterinarians, students and nurses) separately. For each blind classifier, the sum of the evaluations given to each wound, for a specific parameter, was determined. The sums were divided into two groups, since half of the pictures used for the evaluation correspond to wounds treated with Bepanthene® and the other half correspond to wounds where Cyclopray® was applied. Posteriorly, the statistically significant difference between the products was assessed for each parameter, using the sums obtained in the previous step, in order to determine the product best suited for the healing of wounds. In other words, the results obtained were coordinated by parameters, and for each individual parameter they were separated into two groups: Bepanthene® and Cyclopray®.

Every single one of the assessments made by the classifiers was organized and statistically analyzed by the author of this dissertation.

### 3. Results

#### 3.1 Panel 1 - Veterinarians

For the realization of this study, seven veterinarians were asked to evaluate the images collected throughout the duration of this clinical trial.

After organizing the collected data, it's possible to verify that the range of evaluation values, for each parameter, is considerable for both of the products. The greatest difference, between the minimum and maximum classifications, is 338 for the Bepanthene® and 341 for the Cyclopray®, as shown in table 3. The veterinarians possess the most extensive range of values for the evaluations, compared to the veterinary students and nurses.

**Table 3: Range of evaluation values, for each parameter, for Bepanthene® and Cyclopray® in the veterinarians evaluation**

<b>B</b>	<b>Min.</b>	<b>Max.</b>	<b>Dif.</b>
<b>ES</b>	209	445	236
<b>NTT</b>	156	410	254
<b>ET</b>	122	235	113
<b>EA</b>	114	189	75
<b>SCSW</b>	112	390	278
<b>GT</b>	156	494	338
<b>E</b>	298	518	220

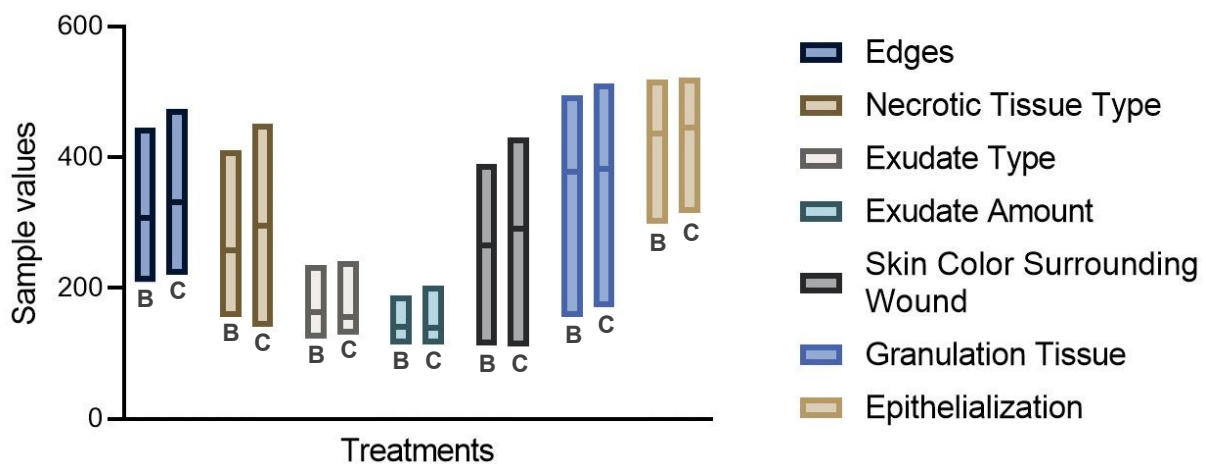
<b>C</b>	<b>Min.</b>	<b>Max.</b>	<b>Dif.</b>
<b>ES</b>	220	474	254
<b>NTT</b>	140	451	311
<b>ET</b>	129	241	112
<b>EA</b>	114	204	90
<b>SCSW</b>	111	430	319
<b>GT</b>	171	512	341
<b>E</b>	314	521	207

Legend: B – Bepanthene®; C – Cyclopray®; Min. – Minimum value of classification; Max. – Maximum value of classification; Dif. – Difference between the minimum and maximum value of classification.

ES – Edges; NTT – Necrotic Tissue Type; ET – Exudate Type; EA – Exudate Amount; SCSW – Skin Color Surrounding Wound; GT – Granulation Tissue; E – Epithelialization.

The distribution of the sample values, for each of the treatments and individual parameters, is illustrated in graphic 1. After careful examination, it's clear that the parameters “Edges”, “Necrotic Tissue Type”, “Skin Color Surrounding Wound” and “Granulation Tissue” present a considerable dispersion of data, a result of unbalanced and uneven evaluation from the classifiers. On the other hand, the parameters “Exudate Type” and “Exudate Amount” display a minor dispersion of data, indicating that the classifiers are somewhat more uniform in their evaluations. The veterinarians present the greatest dispersion of the sample values, when compared to the remaining two panels.

**Graphic 1: Distribution of sample values, for each of the treatments and individual parameters, in the veterinarians evaluation**



Legend: B – Bepanthere®; C – Cyclopspray®.

The horizontal lines on each of the bars represent the mean of the evaluations for each parameter.

The determination of statistically significant differences between the two products, for each parameter, was achieved through the application of a Paired t-test, with the exception of “Exudate Type” and “Exudate Amount”. The sample values for the parameters mentioned previously did not fit the assumption of normality ( $P < 0.05$ ), requiring the use of a Wilcoxon test to perform the statistical analyses.

Table 4 summarizes the results obtained for the classification of the veterinarians, highlighting the relationship between the two products for each parameter. Observing the means of the sums of evaluations, for individual parameters, it's possible to affirm that the Bepanthere® has inferior values compared to the Cyclopspray®, except for the parameters “Exudate Type” and “Exudate Amount”. However, considering the results of the statistical analyses, also presented in table 4, a statistically significant difference ( $P$  value  $< 0.05$ ) was only found between the two products for the parameter “Edges” (Graphic 2). From a statistical standpoint, for the remaining parameters, there was no significant interaction between the products. Nonetheless, even though there is no statistically significant difference between the

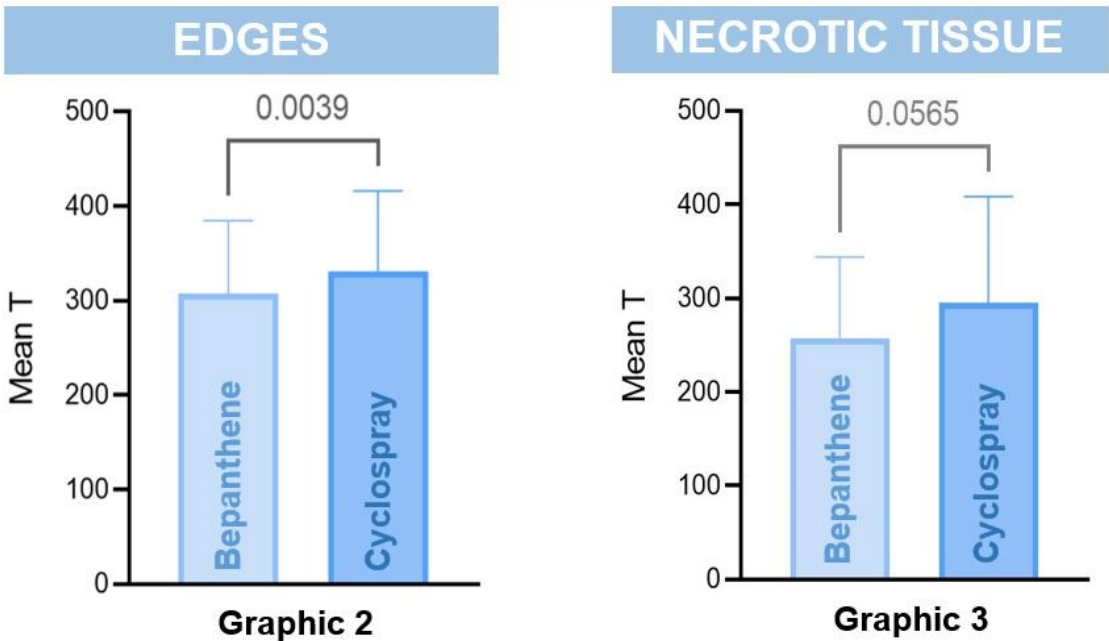
products for the parameters “Necrotic Tissue Type” (Graphic 3), “Skin Color Surrounding Wound” (Graphic 4) and “Epithelialization” (Graphic 5), apparently there is a positive tendency in favor of the Bepanthere® compared to the Cyclospray®, since the P values are close to <0.05.

**Table 4: Relationship between Bepanthere® and Cyclospray®, for each parameter, in the veterinarians evaluation**

Veterinarians	Bepanthere	Cyclospray	P value
Edges	307.1±77.23	330.9±85.16	<b>0.0039*</b>
Necrotic Tissue Type	257.3±86.53	295.3±113.4	0.0565
Exudate Type	162.6±40.32	156.0±38.98	0.6875
Exudate Amount	141.3±27.13	138.6±29.95	0.8750
Skin Color Surrounding Wound	265.7±84.35	290.9±98.39	0.0883
Granulation Tissue	378.1±122.5	381.7±118.7	0.5372
Epithelialization	436.4±72.64	445.0±70.02	0.0835

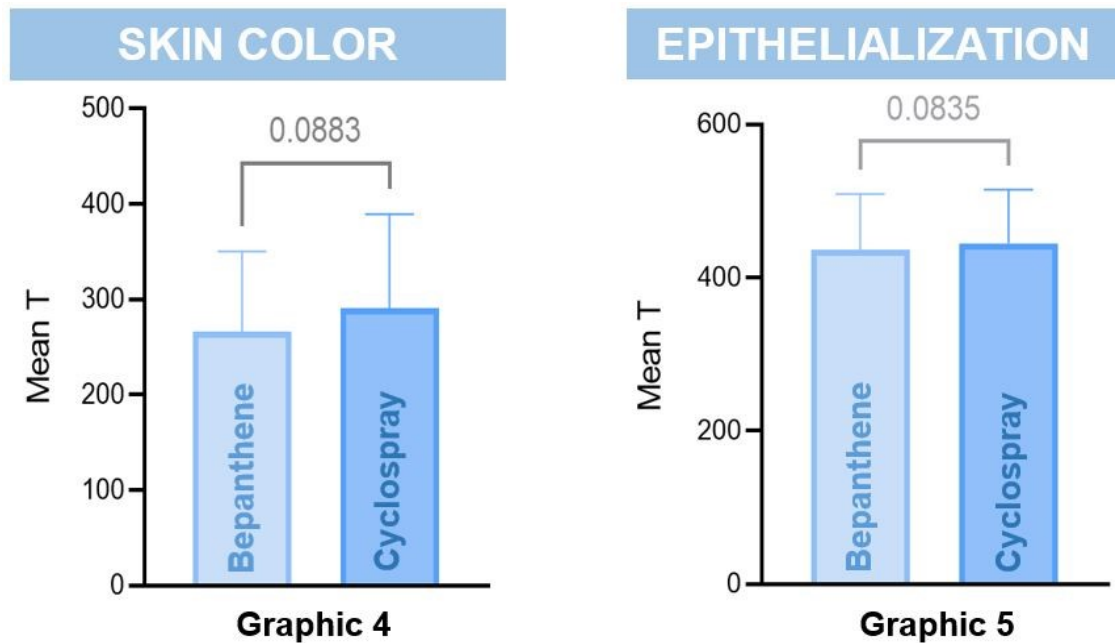
Legend: \* Statistically significant difference (P value <0.05) between the two products for the parameter “Edges”. The values for each of the parameters are presented as means ± standard deviation. The column for the P value characterizes the significance between the two products, Bepanthere® and Cyclospray®.

**Graphic 2 and 3: Relationship between Bepanthere® and Cyclospray® for the parameters “Edges” and “Necrotic Tissue”**



Legend: Mean T – Mean of the sums, for each parameter; statistically significant difference when P value <0.05, present between the two products for the parameter “Edges”.

**Graphic 4 and 5: Relationship between Bepanthen® and Cyclopray® for the parameters “Skin Color Surrounding Wound” and “Epithelialization”**



Legend: Mean T – Mean of the sums, for each parameter; statistically significant difference when P value <0.05.

A ROUT test was executed, in order to determine the presence of outliers among a sample of values. The test detected one outlier (value 241) in the sample of values for the Cyclopray, for the parameter “Exudate Type”. The presence of outliers is liable to cause bias and/or influence estimates.

### 3.2 Panel 2 - Veterinary students

In order to achieve the purpose of this study, five veterinary students were asked to evaluate the images collected throughout the duration of this clinical trial. It is possible to observe, after the analyses of table 5, that the range of evaluation values, for each of the parameters, is noticeable for both products. The largest difference between the minimum and maximum classification is 190 for the Bepanthen® and 171 for the Cyclopray®. The veterinary students possess the least extensive range of values for the evaluations, compared to the veterinarians and nurses.

**Table 5: Range of evaluation values, for each parameter, for Bepanthere® and Cyclopray® in the veterinary students evaluation**

<u>B</u>	Min.	Max.	Dif.
<b>ES</b>	280	376	96
<b>NTT</b>	182	315	133
<b>ET</b>	112	147	35
<b>EA</b>	111	132	21
<b>SCSW</b>	111	245	134
<b>GT</b>	287	477	190
<b>E</b>	318	480	162

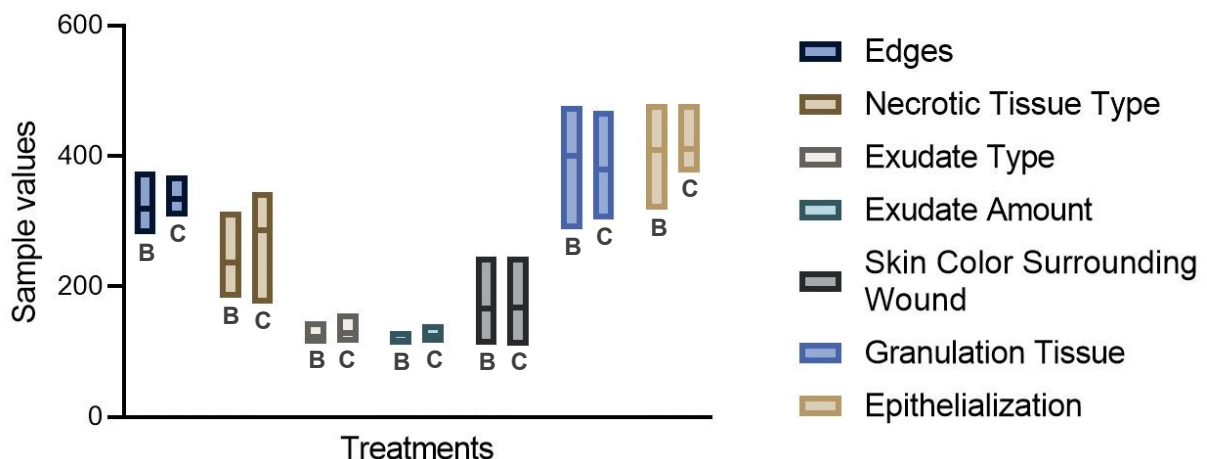
<u>C</u>	Min.	Max.	Dif.
<b>ES</b>	307	370	63
<b>NTT</b>	174	345	171
<b>ET</b>	113	158	45
<b>EA</b>	113	142	29
<b>SCSW</b>	109	246	137
<b>GT</b>	303	469	166
<b>E</b>	374	480	106

Legend: B – Bepanthere®; C – Cyclopray®; Min. – Minimum value of classification; Max. – Maximum value of classification; Dif. – Difference between the minimum and maximum value of classification.

ES – Edges; NTT – Necrotic Tissue Type; ET – Exudate Type; EA – Exudate Amount; SCDW – Skin Color Surrounding Wound; GT – Granulation Tissue; E – Epithelialization;

The distribution of the sample values, for each of the treatments and individual parameters, is illustrated in graphic 6. A closer look reveals that there is very little dispersion in the data, with the parameter “Granulation Tissue” displaying the most noticeable difference in values. On the other hand, the parameters “Edges”, “Exudate Type” and “Exudate Amount” display a minor dispersion of data, indicating that the classifiers are somewhat equal in their evaluations. The veterinary students present the smallest dispersion of sample values, when compared to the other two panels of evaluators.

**Graphic 6: Distribution of sample values, for each of the treatments and individual parameters, in the veterinary students evaluation**



Legend: B – Bepanthere®; C – Cyclopray®.

The horizontal lines on each of the bars represent the mean of the evaluations for each parameter.

The determination of statistically significant differences between the two products for each parameter was achieved through the application of a Paired t-test, since the sample values for every parameter fit the assumption of normality ( $P > 0.05$ ).

Table 6 summarizes the results obtained for the classification of the veterinary students, highlighting the relationship between the two products for each parameter. Observing the means of the sums of evaluations, for individual parameters, it's possible to affirm that the Bepanthere® has inferior values compared to the Cyclopray®, except for the parameter "Granulation Tissue". However, considering the results of the statistical analyses, also presented in table 6, a statistically significant difference ( $P$  value  $< 0.05$ ) was only found between the two products for the parameters "Exudate Type" (Graphic 7) and "Exudate Amount" (Graphic 8). From a statistical standpoint, for the remaining parameters, there was no significant differences between the products. Nonetheless, even though there is no statistically significant difference between the products for the parameter "Edges" (Graphic 9) and "Necrotic Tissue Type" (Graphic 10), apparently there is a positive tendency in favor of the Bepanthere® compared to the Cyclopray®, since the  $P$  values are close to  $< 0.05$ .

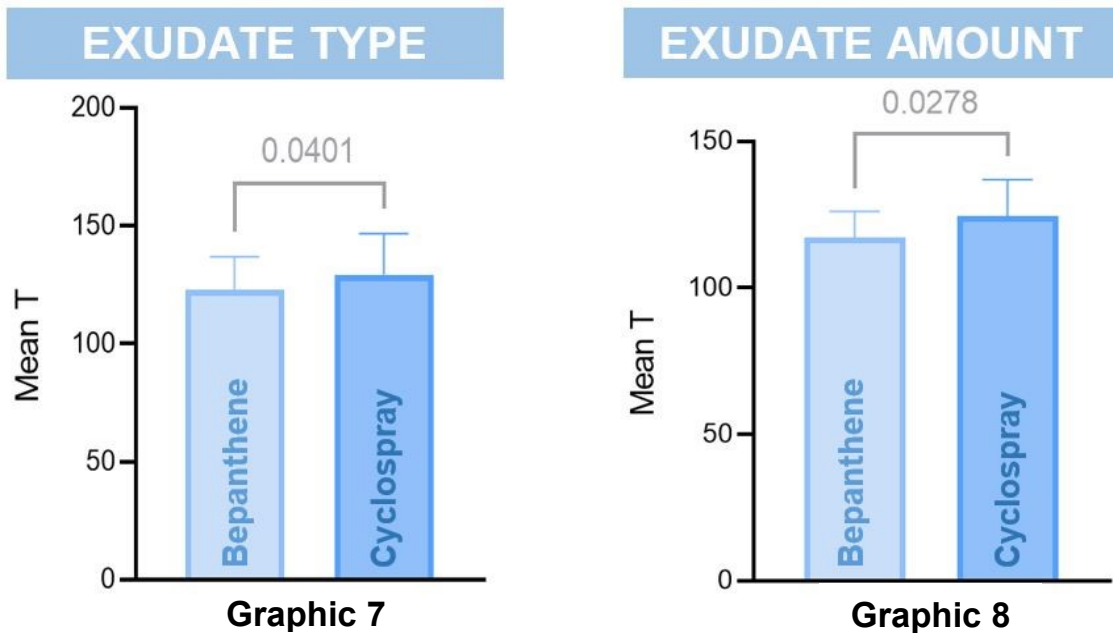
**Table 6: Relationship between Bepanthere® and Cyclopray® for each parameter in the veterinary students evaluation**

<b>Veterinary Students</b>	<b>Bepanthere</b>	<b>Cyclopray</b>	<b>P value</b>
Edges	318.8±37.83	334.2±24.82	0.0598
Necrotic Tissue Type	236.4±56.43	285.6±71.00	0.0658
Exudate Type	122.8±13.97	129.2±17.46	<b>0.0401*</b>
Exudate Amount	117.2±8.871	124.4±12.48	<b>0.0278*</b>
Skin Color Surrounding Wound	166.0±51.98	167.4±52.22	0.6660
Granulation Tissue	399.8±74.43	378.6±61.89	0.3061
Epithelialization	409.0±69.39	410.4±42.18	0.9504

Legend: \* Statistically significant difference ( $P$  value  $< 0.05$ ) between the two products for the parameters "Exudate Type" and "Exudate Amount".

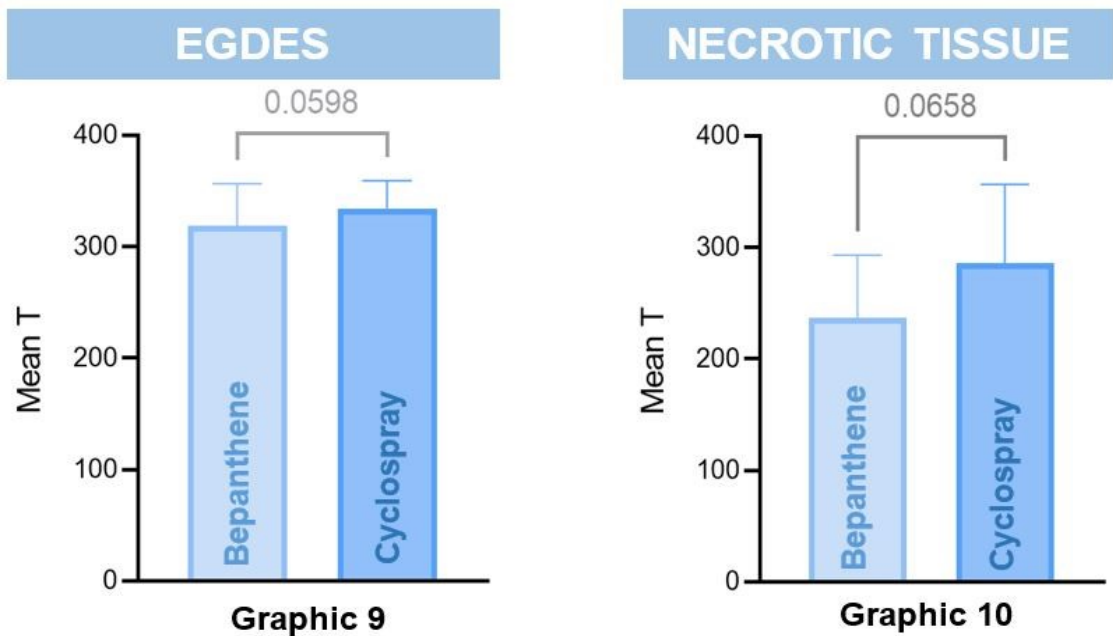
The values for each of the parameters are presented as means  $\pm$  standard deviation. The column for the  $P$  value characterizes the significance between the two products, Bepanthere® and Cyclopray®.

**Graphic 7 and 8: Relationship between Bepanthere® and Cyclopray® for the parameters “Exudate Type” and “Exudate Amount”.**



Legend: Mean T – Mean of the sums, for each parameter; statistically significant difference when P value <0.05, present between the two products for the parameters “Exudate Type” and “Exudate Amount”.

**Graphic 9 and 10: Relationship between Bepanthere® and Cyclopray® for the parameters “Edges” and “Necrotic Tissue Type”**



Legend: Mean T – Mean of the sums, for each parameter; statistically significant difference when P value <0.05.

A ROUT test was executed, in order to determine the presence of outliers among a sample of values. The test did not detect any outlier in the sample values for the parameters, ensuring the power of the statistical tests.

### 3.3 Panel 3 - Nurses from the human medical field

Lastly, five nurses from the human medical field were enlisted in this study, contributing for the evaluation of wounds resulting from the process of disbudding. Considering the data exposed in table 7, it's clear that the range of evaluation values, for each individual parameter, is considerable for the two products. The greatest difference, between the minimum and maximum classifications, is 317 for the Bepanthere® and 300 for the Cyclopray®, as shown in table 7.

**Table 7: Range of evaluation values, for each parameter, for Bepanthere® and Cyclopray® in the nurses evaluation**

<b>B</b>	<b>Min.</b>	<b>Max.</b>	<b>Dif.</b>
<b>ES</b>	218	378	160
<b>NTT</b>	158	269	111
<b>ET</b>	114	159	45
<b>EA</b>	114	194	80
<b>SCSW</b>	139	288	149
<b>GT</b>	165	482	317
<b>E</b>	301	498	197

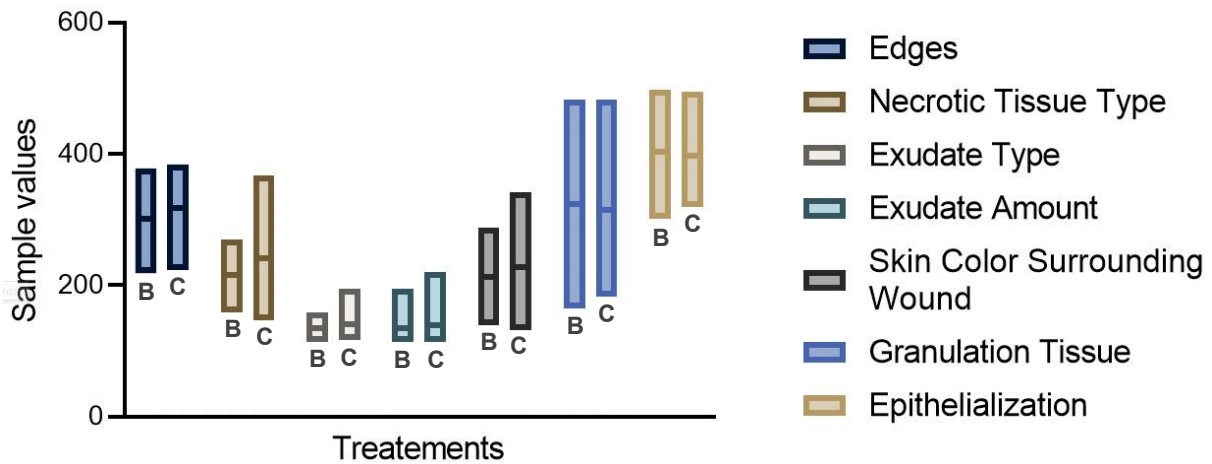
<b>C</b>	<b>Min.</b>	<b>Max.</b>	<b>Dif.</b>
<b>ES</b>	223	384	161
<b>NTT</b>	146	367	221
<b>ET</b>	117	194	77
<b>EA</b>	114	220	106
<b>SCSW</b>	131	341	210
<b>GT</b>	183	483	300
<b>E</b>	319	495	176

Legend: B – Bepanthere®; C – Cyclopray®; Min. – Minimum value of classification; Max. – Maximum value of classification; Dif. – Difference between the minimum and maximum value of classification.

ES – Edges; NTT – Necrotic Tissue Type; ET – Exudate Type; EA – Exudate Amount; SCDW – Skin Color Surrounding Wound; GT – Granulation Tissue; E – Epithelialization.

The distribution of the sample values, for each of the treatments and individual parameters, is illustrated in graphic 11. After careful examination, it's clear that the parameters “Necrotic Tissue Type”, “Skin Color Surrounding Wound”, “Granulation Tissue” and “Epithelialization” present a considerable dispersion of data, a result of unbalanced and uneven evaluation from the classifiers. On the other hand, the parameters “Exudate Type” and “Exudate Amount” display a minor dispersion of data, indicating that the classifiers are more balanced in their evaluations.

**Graphic 11: Distribution of sample values, for each of the treatments and individual parameters, in the nurses evaluation**



Legend: B – Bepanthen®; C – Cyclopray®.

The horizontal lines on each of the bars represent the mean of the evaluations for each parameter.

The determination of statistically significant differences, between the two products, for each parameter was achieved through the application of a Paired t-test, with the exception of the parameter “Exudate Amount”. The sample values for the parameter mentioned previously did not fit the assumption of normality ( $P < 0.05$ ), requiring the use of a Wilcoxon test to perform the statistical analyses.

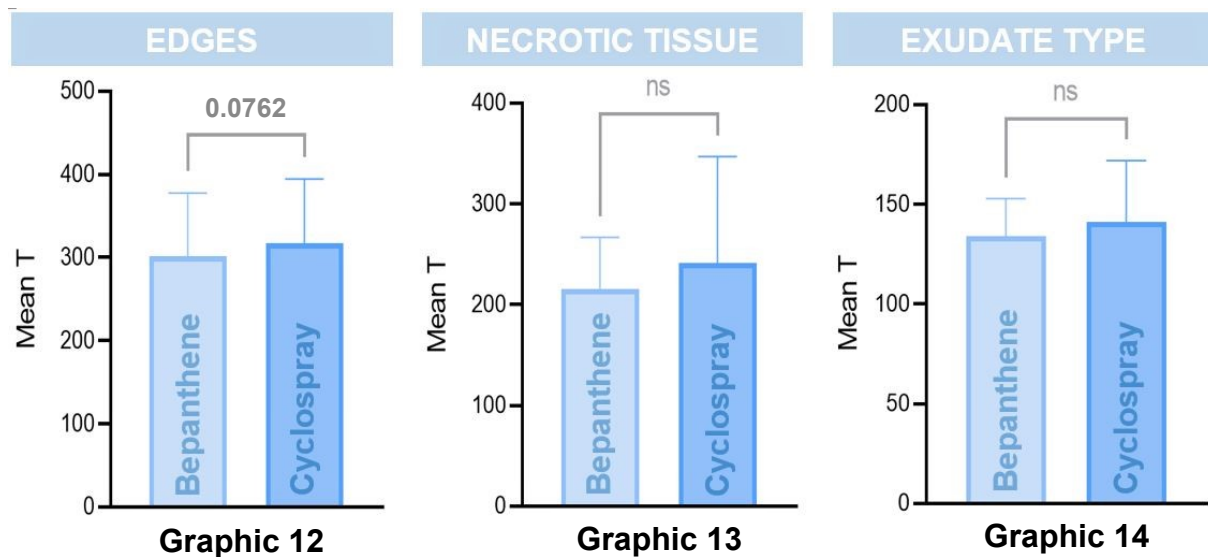
Table 8 summarizes the results obtained for the classification of the nurses, highlighting the relationship between the two products for each parameter. Observing the means of the sums of evaluations, for individual parameters, it’s possible to affirm that the Bepanthen® has inferior values compared to the Cyclopray®, except for the parameters “Granulation Tissue” and “Epithelialization”. However, considering the results of the statistical analyses, also presented in table 8, a statistically significant difference ( $P$  value  $< 0.05$ ) was not found between the two products for any of the parameters. From a statistical standpoint, there was no significant differences in healing between the products (Graphic 13 and 14). Nonetheless, even though there is no statistically significant difference between the products for the parameter “Edges”, apparently there is a positive tendency in favor of the Bepanthen® compared to the Cyclopray®, since the  $P$  value is closer to  $< 0.05$  (Graphic 12).

**Table 8: Relationship between Bepanthen® and Cyclopray®, for each parameter, in the nurses evaluation**

Nurses	Bepanthen	Cyclopray	P value
Edges	301.6±75.79	317.0±77.29	0.0762
Necrotic Tissue Type	215.8±50.91	241.2±105.7	0.3740
Exudate Type	134.0±18.80	141.2±30.65	0.3989
Exudate Amount	134.6±33.64	139.6±45.30	0.6250
Skin Color Surrounding Wound	212.0±68.52	227.0±92.85	0.4672
Granulation Tissue	323.2±144.1	315.0±131.2	0.6213
Epithelialization	403.2±89.13	397.8±69.79	0.7844

Legend: The values for each of the parameters are presented as means ± standard deviation. The column for the P value characterizes the significance between the two products, Bepanthen® and Cyclopray®.

**Graphic 12, 13 and 14: Relationship between Bepanthen® and Cyclopray® for the parameters “Edges”, “Necrotic Tissue” and “Exudate Type”**



Legend: Mean T – Mean of the sums, for each parameter; ns - Not statistically significant; statistically significant difference when P value <0.05.

A ROUT test was executed, in order to determine the presence of outliers among a sample of values. The test detected two outliers in the sample of values, for both the Bepanthen® (value 194) and the Cyclopray® (value 220), for the parameter “Exudate Amount”. The presence of outliers is liable to cause bias and/or influence estimates.

### 3.4 Joint assessment

In order to achieve an overall perception of the evaluations, que classifications given by each group of evaluators were put together, allowing a joint assessment of the best product for the healing of disbudding wounds.

Following the organization of the collected data, it's possible to verify that the range of evaluation values, for each parameter, is considerable for both products. The greatest difference, between the minimum and maximum classifications, is 338 for the Bepanthere® and 341 for the Cyclopray®, as shown in table 9, coinciding with the values obtained for the veterinarians classification.

**Table 9: Range of evaluation values, for each parameter, for Bepanthere® and Cyclopray® in the overall evaluation**

<b><u>B</u></b>	<b>Min.</b>	<b>Max.</b>	<b>Dif.</b>
<b>ES</b>	209	445	236
<b>NTT</b>	156	410	254
<b>ET</b>	112	235	123
<b>EA</b>	111	194	83
<b>SCSW</b>	111	390	279
<b>GT</b>	156	494	338
<b>E</b>	298	518	220

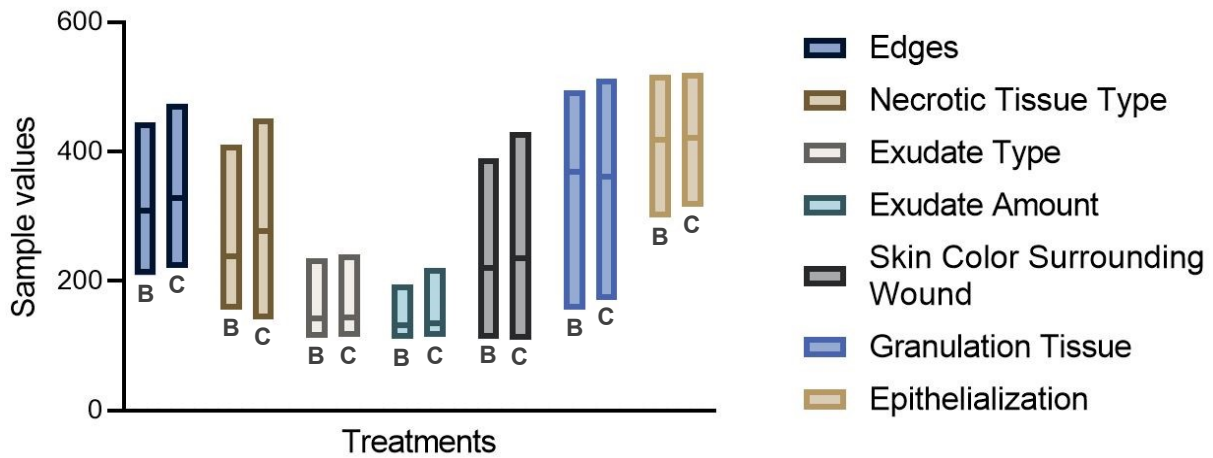
<b><u>C</u></b>	<b>Min.</b>	<b>Max.</b>	<b>Dif.</b>
<b>ES</b>	220	474	254
<b>NTT</b>	140	451	311
<b>ET</b>	113	241	128
<b>EA</b>	113	220	107
<b>SCSW</b>	109	430	321
<b>GT</b>	171	512	341
<b>E</b>	314	521	207

Legend: B – Bepanthere®; C – Cyclopray®; Min. – Minimum value of classification; Max. – Maximum value of classification; Dif. – Difference between the minimum and maximum value of classification.

ES – Edges; NTT – Necrotic Tissue Type; ET – Exudate Type; EA – Exudate Amount; SCSW – Skin Color Surrounding Wound; GT – Granulation Tissue; E – Epithelialization.

The distribution of the values, for each of the treatments and individual parameters, is illustrated in graphic 15. After careful examination, it is clear that the parameters “Edges”, “Necrotic Tissue Type”, “Skin Color Surrounding Wound”, “Granulation Tissue” and “Epithelialization” present a considerable dispersion of data, a result of unbalanced and uneven evaluation from the classifiers. On the other hand, the parameters “Exudate Type” and “Exudate Amount” display a minor dispersion of data, indicating that the classifiers are more balanced in their evaluations.

**Graphic 15: Distribution of sample values, for each of the treatments and individual parameters, in the overall evaluation**



Legend: B – Bepanthen®; C – Cyclopray®.

The horizontal lines on each of the bars represent the mean of the evaluations for each parameter.

The determination of statistically significant differences between the two products for the parameters “Edges” and “Epithelialization” was achieved through the application of a Paired t-test. The values for the remaining parameters did not fit the assumption of normality ( $P < 0.05$ ), requiring the use of a Wilcoxon test to perform the statistical analyses.

Table 10 summarizes the results obtained for the all-panels classification, highlighting the relationship between the two products for each parameter. Observing the means of the sums of evaluations, for individual parameters, it is possible to affirm that the Bepanthen® has inferior values compared to the Cyclopray®, except for the parameter “Granulation Tissue”. However, considering the results of the statistical analyses, also presented in table 10, a statistically significant difference ( $P$  value  $< 0.05$ ) was only found, between the two products, for the parameters “Edges” (Graphic 16) and “Necrotic Tissue Type” (Graphic 17). From a statistical standpoint, for the remaining parameters, there was no significant differences between the products (Graphic 18).

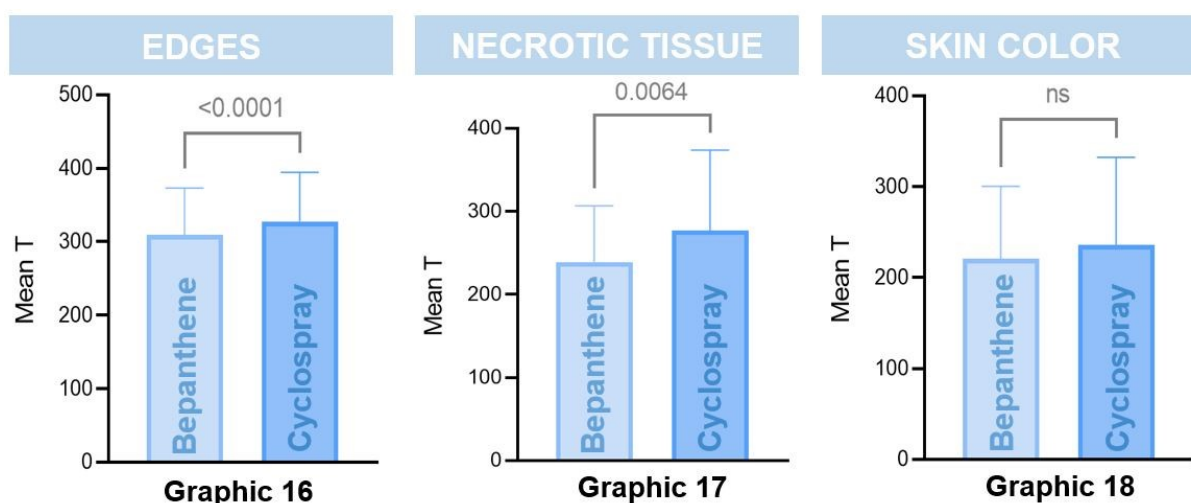
**Table 10: Relationship between Bepanthere® and Cyclopray®, for each parameter, in the overall evaluation**

Nurses	Bepanthere	Cyclopray	P value
Edges	308.9±63,87	327.8±66.49	<0.0001*
Necrotic Tissue Type	238.9±67.59	276.5±97.18	0.0064*
Exudate Type	142.5±32.67	143.8±31.86	0.3344
Exudate Amount	132.2±26.21	134.7±30.59	0.1558
Skin Color Surrounding Wound	220.6±79.77	235.8±96.33	0.1225
Granulation Tissue	368.4±114.9	361.2±107.2	0.8432
Epithelialization	418.6±73.56	420.9±62.90	0.7702

Legend: \* Statistically significant difference (P value <0.05) between the two products for the parameters “Edges” and “Necrotic Tissue Type”.

The values for each of the parameters are presented as means ± standard deviation. The column for the P value characterizes the significance between the two products, Bepanthere® and Cyclopray®.

**Graphic 16, 17 and 18: Relationship between Bepanthere® and Cyclopray® for the parameters “Edges”, “Necrotic Tissue” and “Exudate Type”**



Legend: Mean T – Mean of the sums, for each parameter; statistically significant difference when P value <0.05, present between the two products for the parameters “Edges” and “Necrotic Tissue Type”; ns - Not statistically significant.

A ROUT test was executed, in order to determine the presence of outliers among a sample of values. The test detected various outliers in among the values, specifically in the parameters “Exudate Type” and “Exudate Amount”. For the parameter “Exudate Type”, the Bepanthere® presented one outlier (value 235), and the Cyclopray® also presented one outlier (value 241). However, for the parameter “Exudate Amount”, the Bepanthere®

presented three outliers (values 164, 189 and 194), and the Cyclospray® presented two outliers (values 204 and 220). The presence of outliers is liable to cause bias and/or influence estimates.

### 3.5 “Non-applicable” responses

For every group of classifiers and parameters, each of the ratings “NA” was considered as an intermediate classification, in other words, a rating of three. However, the use of an intermediate rating is liable to cause a deviation in the results, since the rating can be over or undervalued. The percentage of “NA” for each parameter, in the three groups of evaluators, was calculated and presented in Table 9:

**Table 11: Percentage of the evaluation “Non Applicable (NA)” for each parameter**

	Veterinarians		Veterinary Students		Nurses	
	<u>B</u> (%)	<u>C</u> (%)	<u>B</u> (%)	<u>C</u> (%)	<u>B</u> (%)	<u>C</u> (%)
Edges	0.40	0.13	1.12	1.68	3.36	2.62
Necrotic Tissue Type	0.53	1.33	2.24	<b>25.79</b>	1.87	<b>10.84</b>
Exudate Type	0.53	0.93	0.75	2.99	1.31	1.68
Exudate Amount	0	0.53	0.56	2.80	1.31	1.68
Skin Color Surrounding Wound	<b>15.89</b>	<b>16.69</b>	6.54	<b>15.51</b>	2.24	3.18
Granulation Tissue	0.67	1.74	2.99	<b>33.64</b>	2.43	<b>12.71</b>
Epithelialization	0.67	0.4	2.80	<b>21.12</b>	2.24	<b>11.96</b>

Legend: B – Bepanthen®; C – Cyclospray®; % - percentage.

□ Value between 10-20%; □ Value between 20-30%; □ Value superior to 30%.

As shown in the table above, the group of evaluators that classified with “NA” the most was the veterinary students, followed by the nurses from the human medical field. Lastly, the use of this rating is more prominent in the pictures that correspond to wounds treated with Cyclospray® across all groups.

#### 4. Discussion

Considering the rise in antibiotic resistance, in a small part probably associated with the use of topical agents containing antibiotics (Williamson et al. 2017), the need to develop alternative treatments has been rapidly increasing. In this sense, the main objective of this study was to assess the possibility of replacing the antibiotic based spray with the alternative product, Bepanthe®<sup>®</sup>, widely used in human medicine in the treatment of burns and skin irritations (Bayer 2021). The viability of this hypothesis was determined by comparing the healing processes of disbudding wounds, using Bepanthe®<sup>®</sup> or Cyclopray®.

In terms of advantages of the present study, it is important to mention that all of the animals came from the same farm, which means they were subjected to the same environmental conditions, such as handling, feeding, housing, and the procedures performed. The use of a preexisting validated healing scale, Bates-Jensen Wound Assessment Tool, also represents an advantage to the study, since it has been previously successfully used in the determination of wound status. The process of disbudding was accomplished using a cautery, considered the method that allows for better results and more efficient acute pain management, in comparison to other techniques, such as chemical disbudding and amputation (Stafford and Mellor 2011; Stilwell et al. 2007). The disbudding process was accompanied by proper analgesia and local anesthesia (Skarda 1986), safeguarding the welfare of the animals that underwent the procedure. Lastly, the attainment of photographic evidence, organization of data and statistical analyses were all performed by the author of this study, promoting consistency.

For the duration of this study, there were no evident complications, such as exuberant infection of the wounds, loss of appetite or abnormal signs of discomfort related to the procedure and subsequent healing process. However, at the final assessment of the second group, it was noted that the animal 3932 had died, due to an illness unrelated to the process of disbudding (pneumonia).

The evaluation of disbudding wounds healing was accomplished through the observation of images of the injuries, using the Bates-Jensen Wound Assessment Tool for the classification, correlating a higher score with a more severe wound status. The assessment was made by a panel of blind-to-treatment evaluators, constituted by seven veterinarians, five veterinary medicine students, and five nurses from the human medical field. All of the selected participants of the study responded to the classification, generating the data used in the statistical analyses.

Following the organization of the collected data, it is possible to confirm that the veterinarians possessed the most extensive range of values for the evaluations, when compared to the nurses and veterinary students, with the greatest difference, between maximum and minimum classifications, being 338 for the Bepanthe®<sup>®</sup> and 341 for the

Cyclopray®. On the other hand, the veterinary students presented the least extensive range of values for the evaluation, with the greatest difference, between maximum and minimum classification, being 190 for the Bepanthene® and 171 for the Cyclopray®.

Considering the distribution of the sample values, for each of the treatments and individual parameters, the veterinarians and nurses displayed considerable dispersion of the data, result of an unbalanced and uneven classification from the evaluators, while the veterinary students presented minor dispersion, compatible with a somewhat equal evaluation among classifiers. These findings lead to the conclusion that the students were the most consistent in their evaluations, while the veterinarians and nurses presented noticeable discrepancies in their assessment. The composition of the groups is a factor that contributes to discrepancies within the evaluation of a group, since men and women tend to have different outlooks on pain and severity of injuries (Vallerand and Polomano 2000). The veterinarian panel was constituted by five men and two women, the nurse panel was made up of two men and three women, and lastly, the student panel integrated one man and four women. The students presented more uniformity in the constitution of the panel, which may be a factor contributing to the uniformity of the evaluations. Lastly, the differences throughout the assessments of the different panels may be related to the attention to detail. Naturally, veterinarians are used to evaluating different animal wounds, paying more attention to details that the untrained eye cannot capture, and recognizing different patterns related to wound infection. However, veterinary students and nurses from the human medical field are inexperienced in the evaluation of animal wounds, and that reflects in their capacity of assessing wound healing.

Considering the raw values obtained from the classification of the veterinarians, present in table 4, it is possible to affirm that the values obtained for the Bepanthene®, in relation to the Cyclopray®, are lower for every parameter, except “Exudate Type” and “Exudate Amount”. However, after performing a statistical analyses, it is evident that a statistically significant difference ( $P$  value  $<0.05$ ), between the products, only exists for the parameter “Edges” (Graphic 2). For every other parameter, there were no statistically significant differences ( $P$  value  $>0.05$ ) between the products. Nonetheless, for the parameters “Necrotic Tissue Type” (Graphic 3), “Skin Color Surrounding Wound” (Graphic 4) and “Epithelialization” (Graphic 5), there was a positive tendency in favor of the Bepanthene®, since the  $P$  values were close to  $<0.05$ . These findings lead us to conclude that the veterinarians consider the product Bepanthene® superior in the process of healing, when considering the parameter “Edges”, and recognize that the healing potential may be better than the one of the Cyclopray® for the parameters “Necrotic Tissue Type”, “Skin Color Surrounding Wound” and “Epithelialization”.

The values presented by the veterinary students, displayed in table 6, show that the Bepanthere® presents lower values, in comparison to the Cyclopray®, for every parameter, with the exception of “Granulation Tissue”. However, following the statistical analyses, it is possible to affirm that a statistically significant difference, between the two products, is only present for the parameters “Exudate Type” (Graphic 7) and “Exudate Amount” (Graphic 8). In spite of the absence of a statistically significant difference for the other parameters, “Edges” (Graphic 9) and “Necrotic Tissue Type” (Graphic 10) present a positive tendency in favor of the Bepanthere®, since the P values are close to  $<0.05$ . In light of these results, it is possible to conclude that the veterinary students favor the Bepanthere® in the process of healing, when considering the parameters “Exudate Type” and “Exudate Amount”, and highlight the potential of the Bepanthere® to be better at healing than the Cyclopray®, for the parameters “Edges” and “Necrotic Tissue Type”.

When considering the results presented by the nurses from the human medical field, exposed in table 8, the Bepanthere® displays lower result values in comparison to the product Cyclopray®, except for the parameters “Granulation Tissue” and “Epithelialization”. However, the statistical analyses does not show a statistically significant difference, between the products, for any of the parameters, which means that there is no real difference between the products, from a statistical standpoint. Nonetheless, a positive tendency in favor of the product Bepanthere®, compared to the Cyclopray®, was found in the parameter “Edges”, since the P value is close to  $<0.05$ . Although the results do not favor any of the products, the findings lead us to conclude that the Bepanthere® has more healing potential, in relation to the Cyclopray®, for the parameter “Edges” (Graphic 12).

Lastly, the results of the joint assessment, considering the evaluations of the different groups as a total, are exposed in table 10, demonstrating that the Bepanthere® presents inferior values when compared to the Cyclopray®, with the exception of the parameter “Granulation Tissue”. However, considering the statistical analysis, a statistically significant difference, between the products, is only present in the parameters “Edges” (Graphic 16) and “Necrotic Tissue Type” (Graphic 17). From a statistical standpoint, for the remaining parameters, there was no significant differences between the products (Graphic 18). Based on these findings it is safe to assume that, as a whole, the classifiers favor the Bepanthere® in the healing process, when considering the parameters “Edges” and “Necrotic Tissue Type”.

As previously mentioned, the healing-scale was adapted to fit the requirements of this study, by removing the parameters unfit to be evaluated through images and by adding the possibility of a “Non-applicable” rating. However, when considering the statistical analyses, the interpretation of this classification was difficult, since it does not have a defined score that can be added to the final total. The author of this study and Professor Dr. George Stilwell decided to attribute an intermediate score to the neutral rating “Non-applicable”, since the attribution of

a score zero presented a lot of problems that would impact negatively the outcome the study, due to a high discrepancy in the results, leading to huge standard deviations incompatible with the presence of statistically significant differences. However, this intermediate value did not completely eliminate this problem, since the parameter may be over or undervalued, even with an intermediate score. After analyzing the data, it is clear that the group with the higher percentage of “Non-applicable” ratings was the veterinary students, followed by the veterinary nurses, as shown in table 11. These findings corroborate the previously mentioned hypothesis that the least experienced, when considering animal wounds, showed greater difficulties in the process of evaluation when presented with dubious wounds.

The use of the neutral classification “Non-applicable” is more prominent in the wounds treated with Cyclopray® across all groups, possibly due to the coloring agent from the product, a limitation of this study that will be discussed in depth further in this discussion.

The evidence presented in this study leads to the conclusion that the treatment of disbudding wounds with Bepanthene® is a suitable replacement for the antibiotic based spray, Cyclopray®, since it presented an overall better healing index, across all panels, allowing it to be effectively and safely used as a substitute. The use of the Bepanthene® will not only allow a better healing of the injuries resulting from cautery disbudding, but also decrease the use of topical antibiotics in production animals, putting a stop to the development of some antibiotic resistances. From an economic standpoint, the replacement will not negatively impact the farms, since a tube of a hundred grams of Bepanthene® costs approximately the same as a two hundred milliliter can of Cyclopray®, and it can be used on, roughly, forty animals. Concerning the application of Bepanthene®, in order to guarantee optimal use, a portion similar to the size of a hazelnut must be used for the treatment of disbudding wounds.

This study presented many limitations that must be taken into consideration when analyzing the results obtained. One of the most noticeable limitations to this trial, pointed out by various participants, was the classification complexity. The presentation used for the evaluation contained pictures obtained during all the reevaluations, which means, for each animal, there were eight pictures in the presentation, making up a total of two hundred and sixteen (216) pictures to evaluate. The classifiers pointed out the extensive hours spent assessing all cases, admitting that, at times, it became tiring and lead them to be sloppy or rushed with the process. Another problem pointed out by the evaluators was the fact that some of the wounds treated with Cyclopray® presented a blue tint, due to the coloring agent present in the product, making it very difficult to evaluate parameters related with the observation of color, such as “Necrotic Tissue Type”, “Skin Color Surrounding Wound”, “Granulation Tissue” and “Epithelialization”. In response to this problem, the classifiers resorted to the use of a “Non-applicable” rating to classify the parameters they were not sure about or could not properly evaluate, due to the coloring of the wound, creating a new limitation to this study. The use of

the classification “Non-applicable” presents a challenge to the evaluation of the healing process, since the preexisting scale does not equate the use of this score, making the interpretation difficult. The use of an intermediate and neutral score for this classification aids in the decrease of standard deviations, favoring the statistical analyses, but it does not resolve the problem, since the parameter classified with “Non-applicable” may still be over or undervalued, influencing the classification.

Differences throughout the evaluation of the classifiers were abundantly clear. In the beginning of the classification, the use of the classification “Non-applicable” was more prominent when comparing to the end of the classification, indicating that the participants start to get more comfortable with the process as time progresses. Although it is a natural progression, this acclimatization influences the data collected and, consequently, the final results. Another problem directly related with the classification was the easiness in the distinction of the product used for the treatment of the wounds. In spite of asking the participants to ignore the blue coloring agent characteristic of the Cyclospray®, more than half of the first pictures of wounds treated with the product presented the coloration that only fades overtime, capable of unknowingly influence the classifier.

Considering the presented images, it is evident that there are significant differences between them other than the coloration of the spray, such as the lighting and focus. For the duration of this study, the animals were periodically moved between pens as they got older, and these pens present different lighting conditions, heavily influencing the quality of the picture taken. Another problem was the restraining of the animals, made more difficult by the fact that the animals were loose in larger pens, and increasingly bigger and heavier as the reevaluations progressed, making it difficult to restrain them manually and also obtain the pictures, compromising the focus of the image. However, this variation was common to both groups.

Lastly, another limitation may be pointed out, such as the population size considered for the study. A total of twenty-seven (27) calves were used in this study, mainly due to the availability of the animals, since the farm determined the calves that underwent the procedure, and due to scheduling, considering the portion of the internship dedicated to the collection of data was only three months. The program G\*Power (Germany) is a statistical platform of prediction, capable of calculating the ideal sample size, in order to obtain optimal conditions and high statistical power, allowing the attainment of statistically significant differences. Considering the used test for the statistical analyses, a two-tailed Paired t-test, and assuming an effect size of 0.5 and a power of 0.95, the ideal sample size for this study would be fifty-four (54) animals.

Considering the previously mentioned limitations, it is recommended to repeat the study considering the solutions for the various problems: the evaluators must be given the necessary

time to present their classifications, without being rushed; the spray based antibiotic must be replaced for another product without the coloring agent, or proceed with the removal of the blue color upon the first reevaluation, to avoid the wound being colored; eliminate the possibility of the classification "Non-applicable", in order to obtain more consistent and reliable results; the evaluators should be trained or advised to study the healing scale used, as well as the images that will be evaluated, in order to be familiarized with the process of classification before it starts, in hopes of avoiding the learning curve present throughout the evaluation; when taking the pictures used for the presentation, similar conditions of lighting must be ensured, and the animals must be properly restrained; lastly, the sample size should be increased to the value presented previously, in order to obtain statistically significant differences.

## **5. Conclusion**

This study allowed the comparison of the healing process of disbudding wounds treated with Bepanthen® or Cyclo Spray®. To achieve this comparison, the wounds were photographed and presented to a panel of blind evaluators that applied an adapted format of the validated Bates-Jensen Wound Assessment Tool, generating the data used in the statistical analyses.

In the veterinarians evaluation, there was a statistically significant difference (P value <0.05), between the two products, for the parameter “Edges” in favor of the Bepanthen®. The assessment of the veterinary students showed a statistically significant difference, between the products, for the parameters “Exudate Type” and “Exudate Amount”, showing the superiority of the Bepanthen®. The evaluation presented by the nurses showed no statistically significant difference, between the two products, for any of the parameters. Lastly, the joint assessment demonstrated statistically significant differences, between the two products, for the parameters “Edges” and “Necrotic Tissue Type” in favor of the Bepanthen®.

These findings lead us to conclude that the Bepanthen® presented a better healing index, compared to the Cyclo Spray®, allowing it to be safely used as a substitute of the antibiotic based spray.

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## Attachment 1

**Table 1: Adapted Bates-Jensen Wound Assessment Tool.**

Item	Assessment
<b>Edges</b>	1 = Indistinct, diffuse, none clearly visible 2 = Distinct, outline clearly visible, attached, even with wound base 3 = Well-defined, not attached to wound base 4 = Well-defined, not attached to base, rolled under, thickened 5 = Well-defined, fibrotic, scarred or hyperkeratotic
<b>Necrotic Tissue Type</b>	1 = None visible 2 = White/grey non-viable tissue &/or non-adherent yellow slough 3 = Loosely adherent yellow slough 4 = Adherent, soft, black eschar 5 = Firmly adherent, hard, black eschar
<b>Exudate Type</b>	1 = None 2 = Bloody 3 = Serosanguineous: thin, watery, pale red/pink 4 = Serous: thin, watery, clear 5 = Purulent: thin or thick, opaque, tan/yellow, with or without odor
<b>Exudate Amount</b>	1 = None, dry wound 2 = Scant, wound moist but no observable exudate 3 = Small 4 = Moderate 5 = Large
<b>Skin Color Surrounding Wound</b>	1 = Pink or normal for ethnic group 2 = Bright red &/or blanches to touch 3 = White or grey pallor or hypopigmented 4 = Dark red or purple &/or non-blanchable 5 = Black or hyperpigmented
<b>Granulation Tissue</b>	1 = Skin intact or partial thickness wound 2 = Bright, beefy red; 75% to 100% of wound filled &/or tissue overgrowth 3 = Bright, beefy red; < 75% & > 25% of wound filled 4 = Pink, &/or dull, dusky red &/or fills < 25% of wound 5 = No granulation tissue present
<b>Epithelialization</b>	1 = 100% wound covered, surface intact 2 = 75% to <100% wound covered &/or epithelial tissue extends >0.5cm into wound bed 3 = 50% to <75% wound covered &/or epithelial tissue extends to <0.5cm into wound bed 4 = 25% to < 50% wound covered 5 = < 25% wound covered