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Study of hemp derivates as innovative non-conventional antimicrobials in Veterinary Medicine

Studio dei derivati della Canapa come antimicrobici innovativi non convenzionali in Medicina Veterinaria

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Summary

Antimicrobial resistance (AMR) has become a critical global health concern, with bacteria increasingly developing resistance to most antibiotics currently in use. Predictions suggest AMR-related deaths could escalate from 700,000 annually to 10 million by mid-century. Resistance occurs when microorganisms survive antibiotic concentrations that would normally inhibit or kill them, often due to genetic mutations or the acquisition of resistance genes via horizontal gene transfer. This phenomenon is exacerbated by the misuse and overuse of antibiotics in human and veterinary medicine. In companion animals, such as dogs and cats, the widespread use of antibiotics has contributed to the emergence of resistant strains like methicillin-resistant *Staphylococcus aureus* (MRSA), methicillin-resistant *Staphylococcus pseudintermedius* (MRSP) and multidrug-resistant (MDR) Gram-negative bacteria. Close human-animal interactions further facilitate the bidirectional transmission of resistant bacteria.

Companion animals are often treated with antibiotics for infections such as skin wounds, otitis externa, and urinary tract infections, using drugs also critical in human medicine, including β-lactams, aminoglycosides, and fluoroquinolones. This liberal use, often driven by diagnostic uncertainty or empirical treatment practices, has fostered the spread of resistant bacteria, highlighting the need for stricter antibiotic stewardship. MDR pathogens like MRSA, MRSP and *Pseudomonas aeruginosa* pose particular challenges in veterinary dermatology due to their increasing prevalence and resistance to conventional treatments.

As resistance spreads and new antibiotic development stalls, there is growing interest in alternative antimicrobial agents, including plant-derived compounds. Among these, hemp (*Cannabis sativa L.*) has

demonstrated promising antibacterial properties, particularly against MDR pathogens like MRSA and MRSP. These effects are attributed to cannabinoids, such as THC and CBD, which disrupt bacterial membranes and exhibit synergistic antibacterial activity. Hemp extracts act through complex mechanisms, making it harder for bacteria to develop resistance. Although hemp seed oil, produced by cold-pressing hemp seeds, lacks cannabinoids like THC and CBD, its potential antimicrobial effects warrant investigation due to other bioactive compounds it may contain.

Topical cannabidiol therapies are gaining attention in dermatology due to their antibacterial activity, low likelihood of inducing resistance, and additional skin benefits such as anti-inflammatory, analgesic, and wound-healing properties.

The studies conducted during the doctoral course investigate the antimicrobial activity of hemp seed oil against multidrug-resistant (MDR) bacteria associated with canine skin infections, as well as its potential synergistic effects with gentamicin and enrofloxacin, two antibiotics commonly used in veterinary medicine. In addition, these investigations assess the toxicity of hemp seed oil to determine its safety for future clinical use, while also exploring its regenerative properties for wound healing, highlighting its potential as a topical therapeutic agent.

The results of these studies demonstrated that hemp seed oil exhibits antimicrobial activity against strains of S. aureus and S. pseudintermedius. Additionally, promising results were observed when hemp seed oil was combined with gentamicin. The seed oil extract showed no cytotoxicity effects on the tested cell populations and did not promote wound healing.

Chapter 1: Introduction

1.1 Antimicrobial resistance: key concepts

Microbial drug resistance has emerged as one of the most imminent and significant threats to public health. Over the past half-century, the rapid evolution of antibiotic-resistant bacteria has escalated to a critical level, reaching epidemic proportions worldwide [1]. Current projections indicate that within the next 25 years, nearly all bacterial strains may develop resistance to the majority of antibiotics currently in clinical use[2]. Furthermore, experts predict that deaths due to antimicrobial resistance could rise dramatically, from over 700,000 annually to an estimated 10 million by mid-century [3].

A microorganism is considered resistant when it can survive or proliferate in an antibiotic concentration that would typically inhibit or kill other organisms of the same species. In clinical practice, the terms "susceptible" and "resistant" are commonly used to assess the likelihood of a successful antibiotic treatment [4].

Resistance is more likely to develop when a patient is unable to reach the necessary antibiotic concentration to effectively inhibit or eradicate the bacteria [5]. Microorganisms may have intrinsic resistance to an antibiotic or acquire it after exposure [2]. This resistance can arise through genetic mutations or the direct transfer of resistance genes. These genes are frequently carried on plasmids, which are mobile genetic elements, and can spread through processes such as conjugation, transformation via the uptake of free DNA, or transduction, where bacteriophages transfer similar DNA [6]. Genetic material, including antibiotic resistance genes, can rapidly spread even between bacteria of different species [4]. Studies have shown that factors such as heavy metals and biofilm formation contribute to the increased transmission of antibiotic resistance among bacteria [6],

[7]. Resistant bacteria can disperse through various means, facilitating their spread and the potential to cause infections in diverse environments. Although the specific modes of transmission may differ depending on the bacterial species and the surrounding conditions, several common pathways exist through which resistant bacteria can proliferate [2]. The global AMR crisis is further exacerbated by the overuse and misuse of antibiotics in both human medicine and animal care [8]. Extensive antibiotic use in food-producing, companion, and exotic animals accelerates the selection and spread of resistant bacteria, amplifying the threat. AMR undermines the effectiveness of medical treatments and imposes a heavy economic burden on healthcare systems worldwide [9]. Once bacteria acquire resistance, reversing it is rare and requires a significant reduction in selective pressure over time. Within bacterial populations, some carry antimicrobial resistance genes (ARGs), which may not initially cause clinically significant resistance but can decrease bacterial susceptibility. This fosters an environment in which highly resistant mutants can emerge, particularly in the presence of suboptimal antibiotic concentrations [9]. For these reasons, it is essential to assess antibiotic resistance before administering an antibiotic to ensure effective treatment, prevent the development and spread of resistant strains, and optimize therapeutic outcomes. This can be achieved through various methods, such as disk diffusion, broth microdilution, and molecular techniques, which allow for the accurate determination of bacterial susceptibility profiles. These testing methods help identify the most appropriate antimicrobial agents, guide treatment decisions, and minimize the risk of unnecessary or ineffective antibiotic use.

1.2 Antibiotic resistance in companion animals

Antibiotic resistance in companion animals, including dogs and cats, has become an increasingly critical issue within veterinary medicine [10]. The widespread use of antimicrobial agents in veterinary practice, often for prophylactic purposes or to manage infections, has driven the selection and dissemination of resistant bacterial strains. Frequent overuse or inappropriate application of antibiotics, such as their administration without proper diagnostic testing or their application to non-bacterial conditions, exacerbates this issue[11]. The role of pets as significant contributors to the spread of antimicrobial-resistant bacteria has long been underestimated, with much of the attention historically directed toward food producing animals as the primary source of resistant strains [12]. Cats and dogs play a role in the spread of antimicrobial resistance due to the widespread use of antimicrobial agents in their care and their close interactions with humans[13]. In modern society, the population of these companion animals has growth substantially and the human-pet relationship has transformed significantly over time [14]. Pets are increasingly viewed as integral members of the family, further highlighting the potential for close human-animal contact to contribute to the transmission of resistant pathogens. Moreover, in recent years there has been a growing focus on the welfare of small animals, leading to increased investment in veterinary care, particularly in the prevention of infectious diseases [15]. This shift has resulted in the widespread use of antimicrobial agents in pets, especially in dogs. These include drugs specifically licensed for veterinary use as well as critical compounds commonly used in human medicine [16]. In veterinary practice, antibiotics are often prescribed more liberally, influenced by factors such as diagnostic uncertainty, fear of secondary infections, empirical treatment decisions, and pressure from pet

owners, practices that can contribute to inappropriate use. Common classes of antimicrobials used in small animal medicine include penicillins, cephalosporins, macrolides, lincosamides, tetracyclines, chloramphenicol, sulphonamides, aminoglycosides, and fluoroquinolones [17]. In dogs and cats, antibiotics are most commonly administered for conditions like skin and wound infections, otitis externa, respiratory infections, and urinary tract infections (UTIs). Certain infections in dogs, such as pyoderma and some cases of otitis externa, often require prolonged or repeated treatments. For recurrent pyoderma caused by *Staphylococcus pseudintermedius*, cefalexin is a frequently used option, sometimes applied as continuous low-dose or pulse therapy [13]. Companion animals are now recognized as potential reservoirs of antimicrobial resistance due to their close interactions with humans and the extensive use of broad-spectrum antibiotics in their care [18]. This close interaction provides a pathway for the transmission of MDR bacteria between species. Pets can harbor and spread multidrug-resistant pathogens, including to humans, with methicillin-resistant *S. aureus* (MRSA) serving as a notable example [19] [20] While infections caused by MRSA and multidrug-resistant *Staphylococcus pseudintermedius* remain relatively uncommon in pets, the potential for increased transmission is worrisome [19][21][17].Resistant bacteria, such as MRSA, methicillin-resistant *Staphylococcus pseudintermedius* (MRSP), multidrug-resistant Gram-negative bacteria, and ESBL/AmpC-producing *Enterobacteriaceae*, have been found in both healthy and ill pets [13]. If multidrug-resistant (MDR) bacteria are present in household pets and AMR can transfer between animals and humans, the risk of treatment failure increases significantly, posing a serious threat to the health of both humans and animals. This improper use of antibiotics accelerates the emergence of resistance in key pathogens commonly found

in companion animals, including *Staphylococcus aureus, Staphylococcus pseudintermedius,* and *Pseudomonas aeruginosa* [23]. Recent study have highlighted rising levels of resistance to commonly used antibiotics, such as β-lactams, aminoglycosides, and fluoroquinolones, in bacterial isolates from companion animals [24]. Of particular concern is the spread of MDR bacteria, such as MRSA and MRSP, which are increasingly prevalent in veterinary clinical settings [23]. Studies by Guardabassi et al. [13] and Pomba et al. [24] emphasize the bidirectional transmission of AMR bacteria within households, where resistant fecal bacteria can transfer between humans and pets. For example, MRSA, a bacterium originating in humans, can colonize companion animals transiently, with these pets acting as carriers when living with infected or colonized humans [19], [21], [22]. Although antibiotic use in companion animals is lower than in food-producing animals, critical antimicrobials, categorized by the European Medicines Agency (EMA) as "AVOID USE" or "RESTRICT USE," are still commonly prescribed [25]. This contributes to the rise of resistant strains, including methicillin-resistant *S. aureus* and *S. pseudintermedius* in dogs, with coagulase-positive staphylococci increasingly developing resistance to penicillins. The transmission of resistant bacteria and genes from animals to humans occurs through various pathways, further exacerbating the global AMR crisis [26]. Addressing this issue requires a comprehensive understanding of AMR's complexity, which involves a variety of bacterial species, resistance mechanisms, and reservoirs, as well as the intricate dynamics of bacterial transfer between humans and animals. Raising awareness among veterinarians and implementing regulations, such as guidelines for the responsible use of antibiotics, are essential to reduce antibiotic resistance in companion animals. However, the steady rise in resistance shows the importance of ongoing monitoring,

exploring alternative treatments, and creating better diagnostic tools to ensure antibiotics are used correctly and only when truly needed.

1.3 Plant-derived compounds as novel antimicrobial agents

Interest in the antibacterial properties of plants has grown for several reasons, including increased public awareness of the problems caused by the overuse and misuse of synthetic antibiotics [27]. The antibacterial properties of plant-based compounds holds potential not only for medical applications but also for integration into a wide range of everyday products, such as cosmetics. Moreover, the investigation of antibacterial properties in natural fiber plants presents opportunities for innovation. Traditionally, after extraction of primary fibers, the remaining plant material has seen limited applications. However, research into the antimicrobial potential of these by-products may facilitate their enhanced and more sustainable utilization [27].

Plants are rich in biologically active compounds, many of which have demonstrated antibacterial properties [28]. Researchers have extensively studied plants to assess their potential as innovative antibacterial agents. The antimicrobial phytochemicals identified in plants can be broadly categorized into several groups, including phenolic and polyphenols, terpenoids and essential oils, cannabinoids, alkaloids, lectins and polypeptides [29]. Among these, phenolics and polyphenols encompass a wide range of compounds such as simple phenols, phenolic acids, quinones, flavones, flavonoids, flavanols, tannins, and coumarins [30]. Although much of the traditional knowledge about the antibacterial properties of plants is based on anecdotal evidence, scientific research has confirmed the antimicrobial potential of certain plant compounds [31]. Studies reviewing the antimicrobial properties of various plant species have emphasized the promising antibacterial activity of fiber plants,

especially hemp, which as demonstrated considerable potential in this field [27], [32].

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Chapter 2: Hemp (*Cannabis sativa* L.) – An Overview

2.1 Key characteristics of hemp

Hemp, scientifically known as Cannabis, is a genus of annual flowering plants within the Cannabaceae family [33]. While the term “hemp” and “cannabis” are often used synonymously, they generally refer to distinct applications of the plant. “Hemp” is typically associated with its industrial and commercial uses. In contrast, “cannabis” is more commonly linked to its medicinal applications and psychoactive effects[33]. These plants sprout in the spring and typically reach full bloom by late summer, thought the exact flowering period varies among species and environmental conditions. Pollination occurs primarily through wind dispersal, culminating in the production of achenes by autumn. These small, dry fruits, contain a single seed and play a critical role in the propagation of the plant. A defining feature of this plant is its mainly dioecious nature, meaning male and female reproductive structures typically develop on separate individuals, although monoecious forms, where both structures coexist on a single plant, can also occur [34]. Another distinctive trait is the presence of glandular trichomes, microscopic, hair-like structures that produce and secrete a resinous substance. This resin coats the flowers with a layer of whitish microcrystals, giving them their characteristic appearance. Rich in metabolites, the resin serves as a repository for the plant’s active compounds [35]. The composition and concentration of these metabolites vary significantly among genetic strains, collectively forming the plant’s phytocomplex, the full spectrum of bioactive substances it produces [35]. Cannabis includes three main species: *Cannabis sativa L.*, *Cannabis indica*, and *Cannabis ruderalis* [34]. Among these, *C. sativa L.* is the most extensively studied in the medical field due to its adaptability to diverse climates and ease of cultivation. Cannabis

strains are further categorized into three key phenotypes based on their tetrahydrocannabinolic acid (THCA) content. According to European Union regulations, these include [36], [37] :

- Drug strains: THCA levels up to 20%;
- Intermediate strains: THCA levels up to 0,5%;
- Fiber-type strains: characterized by THCA levels below 0,2% and varying concentrations of Cannabidiolic acid (CBDA).

In addition to THCA and CBDA, *C. sativa L.* contains a remarkable chemical diversity. Over 550 distinct compounds have been identified, including a significant increase in new cannabinoids, which have increased from 70 to 115 in recent years. This chemical richness underscores the plant's potential in medicinal and industrial applications [35], [38].
In Europe, *Cannabis sativa L*. varieties can be legally cultivated if they are registered in the EU plant Variety Database of Agricultural species and have a tetrahydrocannabinol (THC) content not exceeding 0,2% (w/w), referred to thereafter industrial hemp [39].

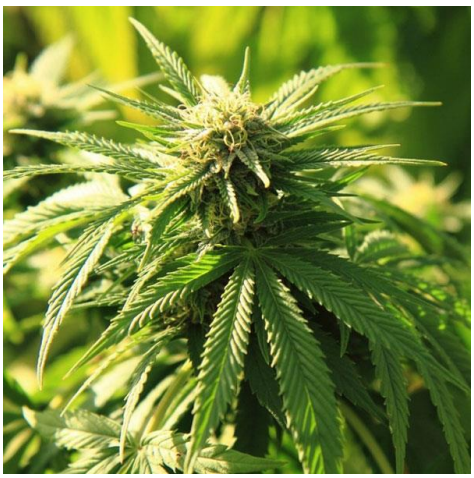

Fig. 1: *Cannabis sativa* L., variety "Futura 75".

2.2 History and uses

Hemp cultivation has long been believed to have its origins in Asia [33]. However, scientific discoveries have uncovered traces of Cannabis in South American mummies, providing evidence that hemp was cultivated in regions beyond the Middle East [40], [41]. This finding challenges traditional assumptions and suggests that the cultivation of hemp was more geographically widespread in ancient times that previously understood. Wild Cannabis thrives in humid area with moderate sunlight and mild temperatures – conditions often found in the environments where nearly human community settled. Due to its natural adaptability to these habitats, nomadic groups living near rivers and streams began observing the plant's growth cycles. Over time, they learned how to domesticate and cultivate hemp, recognizing its value for various uses and integrating it into their daily lives [42]. Wild cannabis is renowned for its robust growth, thriving even in less fertile soils, especially those enriched with nitrogen from animal waste and human byproducts. This remarkable ability to adapt has played a key role in its domestication. Additionally, the plant has proven to be highly versatile, showing a wide array of potential uses across various applications [41].

Hemp is used across a variety of sectors [41]:

- Textile industry: it is used to produce fabrics, canvas, and ropes;
- Construction industry: The woody part of the stem, often considered waste, can be combined with lime to create a bio-composite with excellent insulating properties. This material is ideal for use as filler in walls and as insulation for roofs, interior and exterior walls, and flooring;

- Biodegradable plastics and antibacterial cleaners: the plant's trichomes are valuable in biotechnology, making them key in the production of biodegradable plastics and antibacterial agents;
- Culinary uses: when processed correctly, hemp is sold in various forms, such as flour, oil, seeds, herbal teas, and even beer;
- Raw material for psychoactive substances: it is used to produce hashish (resin extracted from hemp flowers) and marijuana (dried flower clusters of the plants);
- Medical applications: Cannabis has been studied and used by various schools of traditional Eastern medicine, and it continues to be prescribed today for its therapeutic properties. It has proven effective in treating conditions ranging from mild issues like headaches and insomnia to more severe ailments such as chronic pain, psoriasis, glaucoma, and multiple sclerosis;

Over time, the cultivation of domesticated cannabis has grown so significant that it is now beginning to surpass wild hemp species in both demand and production.

2.3 Hemp as a Source of Bioactive Compounds

2.3.1 The phytocomplex

The phytocomplex refers to the entire spectrum of chemical compounds found in a plant, resulting from the specific combinations of its various active constituents. These compounds interact synergistically to give the plant its specific healing properties, which are essential for its medicinal applications [43]. When isolated, individual active compounds may demonstrate diminished effectiveness or produce different effects than when they are part of the whole plant. In cannabis, the phytocomplex encompasses over 800 distinct molecules, including cannabinoids,

terpenes (and terpenoids), flavonoids, and chlorophylls [44]. The interplay between these compounds can generate therapeutic effects that exceed the sum of their individual actions. his phenomenon, known as the "entourage effect," highlights the enhanced efficacy derived from the collective action of the plant's components. The specific ratio and presence of these compounds within each cannabis strain ultimately determine its therapeutic profile and potential side effects [13].

2.3.2 Phytocannabinoids

Phytocannabinoids, also known as plant-derived cannabinoids, are aromatic hydrocarbons containing oxygen and characterized by a terpenophenolic structure [2]. Due to their lipophilic nature, these compounds are nearly insoluble in water. The term "phytocannabinoids" is derived from the Cannabis genus, as these compounds are found exclusively within this plant [12]. The concentration of phytocannabinoids can vary widely depending on factors such as the cannabis strain, the specific plant part being used, and the conditions under which it is cultivated. Cannabinoids are primarily synthesized in glandular trichomes, which are most concentrated in the female inflorescences of *Cannabis sativa L.* [14]. They represent one the most significant products of the secondary metabolism in this plant. Recent studies have identified over 140 phytocannabinoids across various chemovars of *Cannabis sativa* [15]. These cannabinoids exert a range of physiological effects on mammalian tissues through their interaction with the endocannabinoid system. These compounds are generally classified into 10 distinct subclasses [12]. THC and CBD are regarded as "sister" molecules, synthesized by nearly identical enzymes in the cannabis plant, originating from the expression of two alleles at a single gene locus [16]. Genetic variations within Cannabis can result in significant differences in cannabinoid levels, especially THC

and CBD, which can range from less than 0,5% to more than 20%. The highest concentrations of cannabinoids are typically found in the flowers, while the stalks and seeds contain little to no cannabinoids [17]. In general, the concentration of phytocannabinoids varies depending on factors such as tissue type, plant age, variety, growth conditions (including nutrition, humidity, and light exposure), harvest time, and storage conditions. The levels of phytocannabinoids in hemp seeds — and consequently in hemp seed oil —are typically very low, as the seed itself contains only trace amounts of THC and CBD [16]. However, higher concentrations of THC may be found on the outer surface of the seed coat, likely due to contamination from plant leaves or flowers [18]. Cannabinoids levels in the leaves have been shown to decrease with age and along the stem axis, with the highest concentrations found in the leaves of the uppermost nodes [12].

The main cannabinoids are:

- Cannabigerol (CBG);
- Cannabichromene (CBC);
- Cannabidiol (CBD);
- Delta-9-tetrahydrocannabinol (Δ9-THC);
- Delta-8-tetrahydrocannabinol (Δ8-THC);
- Cannabinol (CBN);
- Cannabinodiol (CBDN);
- Cannabicyclol (CBL);
- Cannabielsoin (CBE);
- Cannabitriol (CBT);
- Delta-9-tetrahydrocannabivarin (Δ9-THCV);

The most prominent phytocannabinoids found in Cannabis are THC, CBD, CBG, and CBC [14]. Among these, THC and CBD are the most well-known and extensively studies compounds [17]. It is important to note that in raw cannabis inflorescences, cannabinoids exist in their acidic forms, such as THCA and BCDA. When exposed to specific temperatures, these molecules undergo decarboxylation, losing a carboxyl group (-COOH), which activates them into their bioactive forms, THC and CBD [19]. CBD is also known for its potent anticonvulsant properties. Other cannabinoids, such as Cannabigerol (CBG) and Cannabichromene (CBC), are present in the female inflorescences of hemp and exhibit significant antibacterial activity, along with anti-inflammatory and anti-proliferative effects [14].

2.3.2.1 Cannabidiol (CBD)

Cannabidiol (CBD), the primary non-psychoactive component of cannabis, is a small lipophilic molecule (MW 314 Da) first identified in 1940 as a derivative of cannabidiolic acid [20]. In recent years, CBD has attracted significant interest within the biomedical community for its potent analgesic and anti-inflammatory properties. Unlike Δ9-THC, CBD offers these therapeutic benefits without causing unwanted psychotropic effects [21]. CBD is a phytocannabinoid characterized by a pentyl-substituted bis-phenol aromatic structure (pentylresorcinol) connected to an alkyl-substituted cyclohexene terpene ring system. It is one of over 100 biologically active cannabinoids that can be extracted from the *Cannabis sativa L.* plant [22]. First isolated from Minnesota Wild Hemp in 1940, its complete chemical structure was elucidated only in 1963 [23].

CBD exhibits a remarkable polypharmacological profile and has been extensively investigated across a wide spectrum of clinical indications. Research on cannabinoids has focused on applications such as mitigating

chemotherapy-induced nausea and vomiting, stimulating appetite in HIV/AIDS, and managing chronic pain, spasticity associated with multiple sclerosis or paraplegia, as well as conditions including depression, anxiety disorders, sleep disturbances, psychosis, glaucoma, and Tourette syndrome. Notably, CBD demonstrates significant anti-inflammatory and neuroprotective properties, making it a promising candidate for therapeutic interventions in various pathological conditions [24].

2.3.2.2 Tetrahydrocannabinol (Δ^9-THC)

Tetrahydrocannabinol (Δ^9-THC) is one of the primary bioactive compounds found in *Cannabis sativa* and plays a central role in the plant's pharmacological properties [21]. Δ^9-THC exerts its effects by binding to the cannabinoid receptors CB1 and CB2 within the endocannabinoid system (ECS), thereby modulating various physiological processes [25]. Its biosynthetic precursor is Δ^9-tetrahydrocannabinolic acid A (THCA-A), which is predominantly stored in the glandular trichomes of *C. sativa* flowers and leaves. THCA-A constitutes the majority of total THC in the plant, particularly in its fresh state. As *C. sativa* matures, THCA-A acts as a necrosis-inducing factor, potentially influencing the plant's lifecycle [26], [27]. Through a process of non-enzymatic decarboxylation, triggered by exposure to heat or light, THCA-A is converted into its active form, Δ^9-THC [28]. This transformation can occur naturally during the drying and curing phases of the plant or more rapidly during activities such as smoking, which subjects plant material to high temperatures [21]. THC's effects on neurotransmission are multifaceted and dose- as well as duration-dependent. Acute THC exposure enhances neuronal activity, primarily by increasing dopamine release in the brain's reward pathways. However, prolonged or chronic THC exposure has the opposite effect, reducing dopamine levels and, consequently, neuronal activity, which may

contribute to cognitive and emotional dysregulation [29].The interaction of THC with serotonin systems also exhibits a complex profile. At high THC concentrations, inhibition of serotonin uptake is observed, potentially altering mood and perception. In contrast, chronic THC use appears to increase the maximal velocity of serotonin uptake, a phenomenon that could reflect adaptive changes in serotonergic signaling [30]. These nuanced effects highlight the importance of understanding THC's pharmacodynamics and the potential consequences of its acute and long-term use.

2.3.3 Terpenes and terpenoids

Terpenes are the primary components of plant resins and essential oils, giving the plant its distinctive aroma, flavour, and colour [31]. They represent a large class of aromatic organic hydrocarbons, structurally related to isoprene, and are produced by many plant species [15]. To date, approximately 55,000 terpenes have been identified, classified based on their chemical structure and isoprene units into monoterpenoids, sesquiterpenoids, diterpenoids, and triterpenoids [32]. The variation in their chemical structures influences their diverse biological activities. They play key roles as mediators in ecological interactions, defence mechanisms, and signal transduction, among other cellular processes. In recent years, the importance of terpenes has become increasingly recognized [32]. However, their content is still not typically considered when classifying plants based on their chemical composition [33]. Since nearly all cannabis strains are rich in terpenes, their presence is crucial for developing a comprehensive chemical profile and understanding the associated biological effects. Terpenes are referred to as terpenoids when they undergo oxidation, a process that typically occurs during events such as the drying of flowers. Terpenoids work synergistically with

phytocannabinoids as part of the plant's defence strategy against predators. Notably, antimicrobial properties have been observed in α – and β-pinene, which exhibit activity against both Gram-positive and Gram-negative bacteria, as well as fungi, including *P. aeruginosa*, *E. coli*, MRSA, and *Candida albicans* [4], [15], [32], [33]. The terpenes found in cannabis (over 140) exhibit a wide range of biological activities, playing a role in modulating (either enhancing or mitigating) the effects of phytocannabinoids, other terpenes, and flavonoids. Additionally, they can produce their pharmacological effects directly through the endocannabinoid system [1]. This large family of metabolites has been recognized for its numerous pharmacological properties, including antimicrobial, antiviral, antiparasitic, antifungal, antitumor, anti-inflammatory, and analgesic effects, which are particularly effective due to their synergistic action with cannabinoids [1]. Several studies have highlighted the importance of terpenes, both in terms of the direct action of individual terpenes and the statistically significant differences in effects observed when cannabis is administered without terpenes [15], [32], [33]. The terpenes most commonly associated with physiological activities include:

- Limonene;
- α-pinene;
- Myrcene;
- Caryophyllene;
- Linalool;

2.3.4 Flavonoids

Flavonoids have become increasingly recognized in both nutrition and medicine for their powerful antioxidant properties. These aromatic

polyphenolic compounds share a common chemical structure, but differ in their subclasses based on variations in the basic structure. As one of the largest and most prevalent groups of secondary plant metabolites, flavonoids are known for their significant physiological effects. To date, around 8,000 different flavonoids have been identified, each with unique biological roles [34]. In cannabis, approximately 23 flavonoids have been found, most of which are also present in other plants (such as quercetin, luteolin, and kaempferol), while others, like cannaflavin, are specific to cannabis and contribute to its distinctive scent [34]. Research has shown that cannaflavin A exhibits potent antioxidant and anti-inflammatory properties [35]. It also has other biological effects, including promoting melanogenesis, antiviral activity, and anti-allergic properties. These functions are partially attributed to the synergistic interaction between flavonoids, terpenes, and phytocannabinoids, which together impact the endocannabinoid system. Additionally, flavonoids have demonstrated independent physiological effects that do not rely on the endocannabinoid system [36].

2.4 Hemp derivatives

2.4.1 Hemp seed oil

Hemp seed has long been recognized as a traditional food source, utilized throughout history in various forms, including raw, cooked, or roasted, while hempseed oil (HSO) has served both as a food and medicinal remedy in China for over 3,000 years [37] . Hemp seed is a rich source of essential vitamins A, C, and E, along with beta-carotene and a diverse array of minerals. It contains 20-25% protein, 20-30% carbohydrates, 25-35% oil, 10-15% insoluble fiber, and a wide range of minerals, notably phosphorus, potassium, magnesium, sulphur, and calcium. Additionally, it provides

moderate amounts of iron and zinc, with zinc playing a crucial role as a cofactor in human fatty acid metabolism [38]. The term “hemp seed oil” refers to an oil extracted through cold pressing of industrial hemp seeds. It has a pleasant flavour and offers several distinct advantages over other vegetable oils. It is renowned for its optimal balance, with a 3:1 ratio of two essential polyunsaturated fatty acids (PUFAs)—linoleic and linolenic acids—that are crucial for human nutrition [39]. In addition to its nutritional benefits, hemp seed oil has demonstrated a range of positive health effects, including supporting lipid metabolism, enhancing cardiovascular health, exhibiting immunomodulatory properties, and contributing to the treatment of various dermatological conditions [37], [40]. However, it contains almost no phytocannabinoids, any small amounts found in processed products are usually due to accidental contamination from the plant’s flowers.

2.4.2 Hemp essential oil

Hemp essential oil is produced by glandular trichomes located on the epidermis of the plant’s leaves and, more prominently, on its inflorescences [41]. This oil is rich in bioactive compounds, with terpenes forming the most abundant component of the volatile fraction[42]. In fact, over 100 terpenes and terpenoids have been identified in hemp essential oil [43]. The primary constituents are monoterpenes, and sesquiterpenes, present in both hydrocarbon and oxygenated forms, with diterpenes following in smaller amounts [41]. Each compound in the essential oil contributes its own distinctive fragrance, and their collective composition creates the unique aromatic profile of different strains, which strongly influences consumer preferences [44]. Generally, strains with higher concentrations of monoterpenes are perceived as more pleasant compared to those dominated by sesquiterpenes. Notably, caryophyllene and its

derivative, caryophyllene oxide, exhibit significant anticancer and analgesic properties [45]. The less volatile fraction of hemp essential oil primarily consists of cannabinoids, with cannabidiol (CBD) being the dominant compound [41]. The chemical profile and extraction yield of hemp essential oil can be significantly affected by various factors, including plant genotype, flowering behavior (dioecious or monoecious), cultivation practices, plant density, harvest timing, material processing, and storage conditions. Hemp essential oil, derived from various cultivars, including Futura 75, demonstrates a broad spectrum of potential applications. Its properties, extensively studied, include antimicrobial activity against bacteria and yeast, antibiotic-enhancing effects, and pest-repellent capabilities [43]. Interest in hemp essential oil has grown rapidly in recent years, driven by a growing body of research exploring its chemical composition and biological activities, particularly its antimicrobial and insecticidal properties [46]. Nissen et al. [45]investigated the antimicrobial activity of EO from three industrial hemp varieties against Gram-positive and Gram-negative bacteria, as well as yeasts associated with human commensals and phytopathogens. Their findings indicated that hemp EO can significantly inhibit microbial growth, although the study was limited by the small number of samples tested. The EO derived from the Futura 75 cultivar has also shown promise as an antimicrobial agent, effectively targeting bacterial strains isolated from clinical environments [45]. In another notable study, Marini et al. [47]demonstrated the potential of hemp EO to reduce the virulence of *Listeria monocytogenes*, a major foodborne pathogen, suggesting potential applications in food safety and processing. Despite these promising findings, several limitations remain in the current research. The small sample sizes, incomplete chemical characterization of the EOs, and the

lack of robust correlations between chemical composition and biological activity highlight the need for more comprehensive and systematic studies to fully realize the potential of hemp EO in antimicrobial applications.

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Chapter 3: Antimicrobial activity of hemp (*Cannabis sativa L.*) derivates

3.1 The antimicrobial potential of *Cannabis sativa L.*

The antimicrobial properties of *Cannabis sativa L.* have been recognized across diverse historical contexts, with documented applications in Egyptian medical papyri, traditional African practices, and European folks medicine [1]. In these traditions, Cannabis was employed as an antiseptic agent for the treatment of wounds, dysentery, and malaria [2]. Despite its historical prominence, scientific investigation into the antimicrobial activity of *Cannabis sativa* has been relatively limited, particularly when contrasted with the extensive research into its pharmacological properties, including its antipsychotic, antiepileptic, anxiolytic, neuroprotective, and other therapeutic effects [3]. In recent years, however, renewed scientific interest has yielded significant advancements in understanding the antimicrobial potential of hemp-derived extracts, positioning them as promising candidates for the development of novel antimicrobial agents [4]. Extracts derived from the entire *Cannabis sativa* plant, including leaves, essential oils, seed oil, and cannabinoids, have shown antimicrobial activity against both pathogenic bacteria and fungi [5]. Beyond these well-studied components, the plant contains a variety of bioactive compounds, such as alkaloids, flavonoids, peptides, tannins, and phenols, many of which are recognized for their antimicrobial properties. This rich chemical diversity suggests that the antimicrobial effects of hemp extracts are likely the result of multiple compounds acting together, with synergistic interactions playing a key role in enhancing their overall efficacy [2].

3.2 Cannabis crude extracts

The investigation of *Cannabis sativa*'s antimicrobial potential dates back to 1960, with a pioneering study by Kabelìk et al. [2]. This research

examined different parts of *C. sativa* var. *indica* to assess their effects on Gram-positive and Gram-negative bacteria. The results showed that the extracts were effective in killing Gram-positive bacteria and *Mycobacterium tuberculosis*. However, they had no impact on Gram-negative bacteria and showed no antifungal activity against the fungi and yeast included in the study [2]. Another significant study examined the antimicrobial properties of extracts derived from the leaves and stems of *C. sativa* against *Staphylococcus aureus*. Using disc diffusion assays, the research revealed the presence of a substantial inhibition zone, underscoring the efficacy of the extracts against this pathogen [6]. The antibacterial properties of *Cannabis sativa* leaf extracts have been evaluated against a variety of bacterial strains, including both Gram-positive and Gram-negative species such as *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* [7]. Consistent with earlier research, the findings revealed stronger antimicrobial activity against Gram-positive bacteria, while the effects on Gram-negative strains were moderate or minimal. In another work, the antibacterial activity of dried *C. sativa* leaf extracts was assessed by disc diffusion method on clinical isolates of methicillin-resistant *Staphylococcus aureus* (MRSA), demonstrating growth inhibition across all tested strains [2]. Additionally, a study by Isaqh et al. [8] investigated the antimicrobial effects of leaf and stem extracts on six multidrug-resistant bacterial strains (*S. aureus*, *Bacillus cereus*, *E. coli*, *Klebsiella pneumoniae*, *P. aeruginosa*, and *Proteus mirabilis*) and five fungal strains from the genera *Aspergillus* and *Candida* [8]. The extracts exhibited significant activity against Gram-positive bacteria but showed little to no effect on Gram-negative bacteria or fungi [9]. Furthermore, *C. sativa*

extracts were found to reduce cell viability and inhibit biofilm formation in *S. aureus* [2]

3.3 Cannabis EOs and terpenes

The antimicrobial activity of Cannabis essential oils (EOs) is largely attributed to the presence of cannabinoids such as CBD, THC and CBDV, which remain effective even at low concentrations [10]. These cannabinoids, naturally present in the oils, play a central role in enhancing their antimicrobial properties, often working synergistically to amplify their effects [11]. The antimicrobial properties of terpene compounds such as β-caryophyllene [12], caryophyllene oxide [13], myrcene [14], limonene [15], α-pinene, and β-pinene [16] are well-documented in scientific literature. Studies on EOs have demonstrated their effectiveness against various pathogenic bacteria including *S. aureus* [17]. A study by Nissen et al. [18] specifically evaluated the antimicrobial activity of EOs extracted from the inflorescences of three different *Cannabis sativa* varieties. All tested oils exhibited notable antimicrobial effects, particularly against Gram-positive bacteria, with pronounced activity against genera such as *Enterococcus* and *Streptococcus*. Among the oils studied, one variety, named FUTURA, showed especially promising results against *Clostridia* species [18]. The essential oil derived from a similar variety of *Cannabis sativa L*. (Futura 75) demonstrated notable antibacterial effects against clinically significant Staphylococcus aureus strains, including both multidrug-susceptible and resistant strains [19]. Additionally, the EO exhibited the ability to eradicate biofilms formed by *S. aureus* [19]Moreover, the Futura 75 EO displayed moderate bactericidal activity against *Listeria monocytogenes* and interfered with its virulence factors by reducing motility, invasion capability, and biofilm formation [20]. In a more recent study by Iseppi et al. [11], 17 Cannabis EOs were

chemically characterized and tested for antibacterial activity. While no significant effects were observed against Gram-negative bacteria, the EOs showed strong activity against Gram-positive microorganisms. Some samples achieved minimum inhibitory concentration (MIC) values against *Staphylococcus* spp. that were comparable to or even lower than those of standard antibiotics [11].

3.4 Cannabinoids

The first investigation into the antimicrobial properties of purified cannabinoids derived from *Cannabis sativa*, specifically Δ^9-tetrahydroccabinol (Δ^9-THC) and cannabidiol (CBD), was conducted by Van Klingeren and Ten Ham in 1976 [2]. Their study demonstrated both bacteriostatic and bactericidal effects against *Staphylococcus aureus* and *Streptococcus* species, with effective concentrations ranging from 1–5 μg/mL. Notably, these compounds exhibited no activity against Gram-negative bacteria [2]. Subsequent research in 1981 expanded on the biological activities of other cannabinoids, such as cannabichromene (CBC) and its related analogues. Their findings revealed that CBC and its isomers not only exhibited potent antibacterial activity but also demonstrated mild to moderate antifungal effects. This evidence underscores the potential of cannabinoids, particularly CBC, as candidates for antimicrobial development [2].

In the context of antimicrobial resistance, Appendino and colleagues [21]conducted a comprehensive investigation of the five major cannabinoids, CBD, CBC, CBG, Δ^9-THC, and CBN, along with related derivatives, against multidrug-resistant *S. aureus* strains. Their findings underscore the potential of cannabinoids as promising alternatives or adjuncts in the fight against resistant bacterial pathogens. All tested cannabinoids demonstrated potent antibacterial activity, with minimum

inhibitory concentration (MIC) values ranging from 0.5 to 2 μg/mL. Notably, a correlation was observed between the chemical structures of cannabinoids and their antibiotic activity, suggesting the involvement of specific interactions with bacterial targets. These findings point to a structure-activity relationship that could inform the design of targeted antimicrobial agents [21].

Building upon these results, Farha et al. [22] further investigated the antibacterial properties of cannabinoids, including CBC, CBD, CBG, CBN, Δ^9-THC, and their precursors. Their study confirmed the potent antibacterial activity of these compounds against methicillin-resistant *Staphylococcus aureus* (MRSA). Moreover, cannabinoids were shown to inhibit MRSA biofilm formation, eradicate pre-formed biofilms, and effectively target stationary-phase cells that exhibit persistence against conventional antibiotics. These results highlight the multifaceted antimicrobial potential of cannabinoids, particularly in addressing biofilm-associated infections and antibiotic resistance [22].

Recent studies have further highlighted the antibacterial properties of CBD, cannabidiolic acid (CBDA), and cannabichromenic acid (CBCA) against Gram-positive bacteria, including methicillin-resistant *S. aureus* (MRSA) [23], [24]. Cannabinoids have also shown synergistic interactions with conventional antibiotics, enhancing their effectiveness against resistant bacterial strains. Conversely, certain antibiotics have been found to amplify and extend the antimicrobial activity of cannabinoids [22], [25], [26].

For example, CBD significantly enhanced the antibacterial activity of erythromycin and rifampicin against *E. coli* and increased the efficacy of kanamycin against *S. aureus*. Additionally, CBD potentiated the action of bacitracin against MRSA, *Enterococcus faecalis*, *Listeria monocytogenes*,

and methicillin-resistant *S. epidermidis* (MRSE), reducing the minimum inhibitory concentration (MIC) of bacitracin by at least 64-fold. These findings underscore the potential of cannabinoids as adjuvants in combination therapies aimed at combating antibiotic-resistant pathogens [26].

Time-kill assays have demonstrated that the combination of cannabidiol (CBD) and bacitracin exhibits synergistic and bactericidal effects [26]. Synergistic antimicrobial effects were also observed between cannabigerol (CBG) and polymyxin B against multidrug-resistant clinical isolates of Gram-negative pathogens, including *Acinetobacter baumannii*, *E. coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa [22]*

However, it is important to note that positive interactions between pure cannabinoids and conventional antibiotics are not universally observed. For example, recent synergy tests revealed that CBD displayed an indifferent effect when combined with certain antibiotics against methicillin-resistant *Staphylococcus aureus* (MRSA) [23]. These findings suggest that the effectiveness of cannabinoid-antibiotic combinations may depend on the specific compounds and bacterial targets involved, warranting further investigation into their mechanisms of interaction.

3.5 Potential antimicrobial mechanisms of Hemp extracts

Although the precise antibacterial mechanism of action of cannabinoids remains unclear, recent research has shed light on potential mechanisms. One proposed mode of action for compounds derived from *Cannabis* involves the disruption of membrane permeability. For example, the terpene limonene has been shown to compromise the integrity of the cell membrane and wall structure in *Listeria monocytogenes*, resulting in the leakage of intracellular components [27]. Similarly, β-caryophyllene has

demonstrated comparable effects, inducing membrane disruption in *Bacillus cereus* [12].

Cannabigerol (CGB) has also been shown to target the cytoplasmic membrane of Gram-positive bacteria. Additionally, the permeabilization of the outer membrane in Gram-negative bacteria significantly reduced the minimum inhibitory concentration (MIC) of CBG for these pathogens (from >128 to 1 μg/mL). This alteration enabled CBG to exert its effect on the inner membrane in a manner similar to its action in Gram-positive bacteria [22].

Microscopic analysis of the effect of cannabichromenic acid (CBCA) on *Bacillus subtilis* revealed significant alterations in both the bacterial membrane and nucleoid, ultimately leading to cell lysis [24]. Similarly, cannabidiol (CBD) demonstrated membrane-related activity, inducing depolarization of the cytoplasmic membrane and disrupting the membrane potential in *S. aureus* [25].

Moreover, the combination of CBD with bacitracin resulted in defects in cell division and irregularities in the cell envelope. These effects were likely due to the downregulation of a critical cell division gene, *ezrA* [26].

A proposed mechanism of antimicrobial action of CBD is the inhibition of outer membrane vesicle (OMV) release. This phenomenon, observed in *E. coli* but not in *S. aureus*, prevents the release of vesicles that play a crucial role in various biological processes. These vesicles contain enzymes such as beta-lactamases, which degrade antibiotics, and are also involved in the horizontal transfer of antibiotic resistance genes [25].

3.6 *In vivo* effects of hemp derivates: interaction with immune system and toxicity

The consideration of cannabis compounds as potential antimicrobial agents raises several concerns, particularly regarding their in vivo efficacy,

interactions with the immune system, pharmacokinetics, adverse effects, and toxicity [2]. For a therapeutic intervention to be successful, drugs must achieve a concentration that exceeds the minimum inhibitory concentration (MIC) for antimicrobial activity, while remaining below levels that could cause toxicity. Additionally, the effectiveness of antimicrobial therapies is not solely dependent on the MIC value; the immunomodulatory properties of the compounds also play a critical role in determining therapeutic outcomes [28].

3.6.1 Cannabinoids and Immune system

The connection between the endocannabinoid system and the immune system, as well as the effects of exogenous cannabinoid ligands on immune function during infections, is not yet fully understood 82. However, various components of the endocannabinoid system have been implicated in immune modulation. These include the regulation of hematopoietic stem and progenitor cell migration, the modulation of adaptive immunity, which affects the activity of T cells and B cells [29]. However, inconsistencies have been observed across studies, with some reporting inhibitory effects on the immune system, while others suggest a stimulatory action on immune cells [2]. These discrepancies are likely due to the complexity of the endocannabinoid network, the diversity of cannabinoid types and their varying effects, differences in experimental methods and protocols, as well as the biphasic nature of cannabinoid responses. Specifically, cannabinoids may exert stimulatory effects at nanomolar concentrations and inhibitory effects at micromolar concentrations [2].

Several studies have demonstrated that Δ^9-THC modulates the immune system, leading to suppressed cellular function and reduced cytokine production, particularly interferon-γ and interleukin-12, which in turn

increases mortality in mice infected with *Legionella pneumophila* [30]. However, cannabinoids have also shown beneficial effects in certain infection models, particularly concerning proinflammatory cytokine levels. For instance, in an animal model of meningitis, the administration of CBD (10 mg/kg) for nine days following a *Streptococcus pneumoniae* challenge prevented memory impairments in rats [31].

Similarly, protective effects were observed in a sepsis animal model where CBD treatment improved cognitive function and reduced mortality in rats [32]. In a murine model of systemic infection with *Methicillin-resistant Staphylococcus aureus* (MRSA), Farha et al. [22] reported that CBG significantly reduced bacterial burden in the spleen at a dose of 100 mg/kg, with results comparable to those of vancomycin at similar doses.

3.6.2 Cannabinoids Toxicity

Toxicity represents one the primary limitations to the use of cannabinoids. In animal models, high doses of Δ^9-THC have been associated with hypothermia, reduced locomotor activity, catalepsy, and antinociception [33].

In rats, the median lethal dose (LD50) of orally administered Δ^9-THC is estimated to range from 800 to 1900 mg/kg, whereas in dogs and monkeys, doses up to 3000 and 9000 mg/kg, respectively, have been shown to be nonlethal [2]. In contrast, cannabinoids such as CBD, CBG, CBC, Δ^9-tetrahydrocannabivarin (Δ^9-THCV), CBDV, and their acidic forms have demonstrated minimal or no psychotropic activity, leading to better tolerance profiles [34]. Pharmacokinetic studies in mice and rats, involving single doses of cannabinoids administered both intraperitoneally and orally at doses of 120 mg/kg (CBD and CBG), 60 mg/kg (CBDV), and 30 mg/kg (Δ^9-THCV), revealed no signs of acute toxicity [35].

In dogs, escalating doses of CBD up to 62 mg/kg were well tolerated, with only mild adverse effects, predominantly gastrointestinal, observed compared to the placebo group [36]. In humans, chronic use of CBD at doses up to 1500 mg/day has been reported to be well tolerated [3]. A report from the WHO Expert Committee on Drug Dependence concluded that CBD is generally well tolerated and possesses a favourable safety profile, recommending that it should not be included in the International Drug Control Conventions [37].

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Chapter 4. Bacterial pathogens in canine pyoderma and otitis externa: an overview

4.1 Pyoderma and Otitis externa in dogs

Pyoderma is a bacterial skin condition that arises from an overgrowth of the skin's normal resident or transient flora. This infection often develops secondary to various factors, including local trauma, excessive scratching, inadequate grooming, seborrhea, parasitic infestations, hormonal imbalances, etc.

Pyoderma can be classified based on the depth of infection [1]:

- Surface pyoderma: includes conditions such as acute moist dermatitis (hot spots), fold pyoderma (intertrigo), and bacterial overgrowth syndrome, which presents as erythema with high bacterial counts but no additional clinical signs;
- Superficial pyoderma: involves the follicular opening and epidermal tissue;
- Deep pyoderma: less common but more severe, penetrating into dermis and carrying a higher risk of bacteraemia. This form is often linked to underlying conditions or immunodeficiencies;

Pyoderma is characterized by symptoms such as pain, crusting, foul odor, and the discharge of blood and pus [1]. It is a leading cause of antimicrobial use in small animal veterinary practice. However, despite its prevalence, canine pyoderma is frequently misdiagnosed, resulting in suboptimal treatment [2]. Skin cytology serves as a crucial diagnostic tool, enabling the detection of inflammatory cells and bacteria associated with pyoderma [3]. Additionally, this technique is valuable for identifying *Malassezia* dermatitis, a common co-occurring infection. Bacterial culture and antimicrobial susceptibility testing are crucial for cases of recurrent pyoderma, as they help address the rising prevalence of resistant infections

[4]. These tests are essential for selecting the most effective systemic antimicrobial treatment. Coagulase-positive staphylococci are widely recognized as the primary cause of pyoderma. While infections were once solely attributed to *S. aureus*, advancements in microbiological techniques have led to the identification of other species, including *S. pseudintermedius* [5]. This species is now known to be the most common pathogen involved, especially in cases of superficial pyoderma. Infections occur due to a complex interplay of genetic, environmental, and immunological factors. Various predisposing conditions and primary causes can trigger the transition of *S. pseudintermedius* from a harmless commensal to a pathogenic bacterium [6]. Other staphylococcal species, such as *S. aureus*, *S. schleiferi*, and *S. hyicus*, may account for up to 10% of pyoderma cases [7]. The increasing prevalence of methicillin-resistant (MRSA) and multidrug-resistant (MDRS) staphylococci has made treating canine pyoderma more challenging [8]. The empirical choice of systemic antibiotics is becoming progressively difficult, and therapy should now be guided by bacterial culture and susceptibility testing, especially in cases of superficial pyoderma unresponsive to empirical treatment, in animals with a history of MRSA, and in all instances of deep pyoderma [9].

Otitis externa is an inflammatory condition affecting the external ear canal, including the ear pinna, and is a leading reason for veterinary visits in small animals, particularly dogs [10]. This condition can be either acute or chronic and may affect one or both ears. Diagnosing otitis externa involves a thorough evaluation, including palpation of the ear canal, visual inspection, otoscopic examination, and cytological analysis of the ear discharge [11]. Changes to the ear pinna can include alopecia, excoriation, crusting, erythema, and hyperpigmentation. The external ear canal may show signs of hyperaemia, ulceration, ceruminous or purulent discharge,

masses, and stenosis, among other abnormalities [10]. Cytological examination of otic contents is the most valuable diagnostic tool for diagnosing and guiding the treatment of otitis externa. It also plays a key role in monitoring the response to therapy [10]. In some cases, bacterial culture samples taken from the horizontal ear canal may be necessary to identify the appropriate treatment and inform the selection of systemic antibiotics, if required [12]. Effective treatment of ear infections involves not only addressing the infection and inflammatory changes but also identifying and treating the underlying causes that contributed to the development of otitis. Topical therapy is the primary treatment for otitis externa, although systemic anti-inflammatory and/or antimicrobial therapies may be necessary for certain patients [13]. While cytological analysis is very helpful for guiding treatment decisions and monitoring progress, simply treating the ear infection alone does not always guarantee a successful outcome. Cleaning the ears before applying topical therapy is essential for reducing otic cerumen, which enhances the effectiveness of the treatment [14]. Additionally, ear cleaning helps disrupt biofilms that may shield bacterial colonies, allowing for better penetration and efficacy of antimicrobial therapy [13]. The most commonly isolated bacteria from the ear canals of dogs with otitis are *Staphylococcus* species. Other bacteria frequently associated with otitis include *Pseudomonas*, *Proteus*, *Enterococcus*, *Streptococcus*, and *Corynebacterium*. Some bacteria, such as *Staphylococcus* spp. and *Pseudomonas* spp., are capable of producing biofilms, which can contribute to persistent infections despite appropriate treatment [10], [15].

4.2 *Staphylococcus aureus*

Staphylococcus aureus (*S. aureus*) was first identified by Alexander Ogston in the late 19th century, when he discovered it in pus from a leg abscess [16]. *S. aureus* is a Gram-positive bacterium characterized by its spherical (cocci) shape and distinctive clustering pattern, often resembling grape-like formations. This microorganism can grow in environments with up to 10% salt concentration and typically produces golden or yellow colonies on culture media, a characteristic that reflects the Latin meaning of its name, "aureus", which means "golden" [17]. *S. aureus* can grow both aerobically or anaerobically (facultative) and at temperatures between 18°C and 40°C. Typical biochemical tests employed for the identification include catalase positive (all pathogenic *Staphylococcus* species) and coagulase positive (to differentiate *S. aureus* from other *Staphylococcus* species) and mannitol fermentation (to distinguish from *S. epidermidis*) [18]. Its pathogenicity is considered "multifactorial", meaning that no single factor is solely responsible for its ability to cause disease. Many of these virulence traits are encoded by "accessory genetic elements", genes located on plasmids, bacteriophages, transposons, or genomic island, rather than within the core genome. These elements are present in some strains but absent in others, resulting in variability in the virulence gene profiles of clinical isolates, which can significantly influence their capacity to cause infections [19]. Recent research has revealed that *S. aureus* colonization in humans is more widespread than previously recognized [20]. It is a major cause of opportunistic infections, ranging from superficial skin conditions to severe systemic diseases, including pneumonia, endocarditis and sepsis. In veterinary medicine, *S. aureus* is particularly relevant in companion animals like dogs, where it is associated with skin and soft tissue infections, otitis externa, surgical site infections,

and wound infections [21]. Transmission occurs through direct skin-to-skin contact or contact with contaminated surfaces, making carriers crucial in the spread and persistence of *S. aureus* strains [17]. As a major cause of infections, *S. aureus* affects a wide variety of animal species, posing significant risks to public health. Over time, the bacterium has adapted to new hosts through host-switching events, acquiring or losing mobile genetic elements, as well as accumulating host-specific mutations, which help it thrive in different populations [22]. Close contact between humans and animals plays a key role in these host-switching events. In fact, strains of *S. aureus* found in companion animals are often derived from humans, with transmission occurring between pet owners and their pets [23]. Dogs and cats are not typically colonized by *S. aureus* but they can occasionally form transient associations with the bacterium, which in some cases can lead to severe infections [23]. In fact, although *S. aureus* is capable of colonizing the healthy canine hair coat, the frequency of its isolation from dogs and cats is generally low, with the bacterium typically recovered from less than 10% of samples [24]. *S. aureus* is a widely studied model of bacterial virulence, characterized by a diverse array of potential virulence factors. Its zoonotic potential further elevates its importance, as pets can serve as reservoirs and vectors for multidrug-resistant strains, including MRSA, posing a risk to humans in close contact. The clinical management of *S. aureus* infections requires careful antimicrobial stewardship, supported by bacterial identification and susceptibility testing, to mitigate the growing challenge of antibiotic resistance [25].

4.3 Methicillin-resistant *S. aureus* (MRSA)

A major concern with *S. aureus* is its remarkable ability to develop resistance to antimicrobial agents, posing a significant challenge to treatment and control efforts. An example of this resistance is methicillin-

resistant *S. aureus* (MRSA), which has become a serious public health threat worldwide [19]. In particular, MRSA is highly problematic because, beyond its inherent resistance to nearly all β-lactam antibiotics, it has a pronounced ability to acquire resistance to other, unrelated classes of antimicrobials. This includes glycopeptides, which are often considered the last line of defence against multidrug-resistant MRSA strains [26]. Methicillin resistance is linked to the presence of the *mecA* gene [22]. This gene encodes PBP2a, an altered penicillin-binding protein of 78 kDa with a very low affinity for β-lactam antibiotics, rendering these drugs ineffective [16]. As a result, methicillin-resistant staphylococci are resistant to a wide array of β-lactam antimicrobials, many of which are critical for treating bacterial infections. Evidence suggests that methicillin-sensitive strains of S. aureus acquired methicillin resistance through the horizontal transfer of SCCmec element, likely originating from coagulase-negative staphylococcal species [27]. This process appears to have occurred independently on multiple occasions. MRSA strains can be classified using both phenotypic and molecular techniques. Phenotypic methods involve examining colonial characteristics, biochemical reactions, antibiotics susceptibility patterns, phage susceptibility, and toxin production. The most widely used molecular typing methods today include pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST), SCC*mec* typing and *spa* typing [24]. The first cases of MRSA were reported in 1961 by two groups in United Kingdom, just a few years after penicillinase-resistant β-lactam antibiotics were introduced, showing how rapidly the bacterium can adapt to new treatments [20], [28]. MRSA in puppies was first identified in 1994, but its widespread occurrence was not well documented until 1999[29], [30], [31]. Cases of canine MRSA infections have since been reported in countries such as Canada and the

Netherlands [6]. While pets can act as carriers of MRSA, posing a potential risk to their owners – particularly those with heightened susceptibility to infections – they are not considered the primary reservoir of MRSA. Instead, pets typically acquired the bacterium through contact with infected humans and serve as a minor secondary reservoir [23]. The role of companion animals as reservoirs of MRSA is still unclear, as it is uncertain whether they act as long-term carriers or simply as contaminated vectors [7]. A “reservoirs” would imply that the host can maintain the pathogen over time, but this has not been proven for companion animals. Some studies suggest that MRSA carriage in these animals is not sustained for long periods, particularly in clean environments. For example, a study shown that MRSA was eradicated in 16 healthy rescue dogs after daily cleaning and disinfection of the kennel, without the administration of antibiotics [32]. Similarly, in a Canadian study, strict hygiene and decolonization of human carriers led to the disappearance of MRSA from all horses on a farm within six months, even without treating the animals [33]. In contrast, MRSA persisted in one dog for weeks, likely due to ongoing contamination from human owners with open wounds [34]. While *S. aureus* may infect cats more often than dogs or horses, no data on MRSA persistence in cats is available [35], [36] . In contrast, pigs are recognized as true reservoirs for MRSA ST398, as it spreads quickly among them and is more common in pigs than in humans [30], [37] In companion animals, MRSA primarily causes skin and soft tissue infections. The most frequently reported conditions include wound infections, surgical site infections, pyoderma, otitis and urinary tract infections[33]. However, opportunistic infections can also arise in various other body sites. Similar to other species, a small percentage of healthy dogs may carry MRSA asymptomatically [38]. In recent years, MRSA together with methicillin-

resistant *Staphylococcus pseudintermedius* (MRSP) have gained recognition as growing concerns in veterinary medicine, with increasing reports of cases, particularly in small animal and equine practices [39]. These resistant strains are significant not only for the health of animals, but also for their potential impact on public health, as they can spread between animals and humans.

4.4 *Staphylococcus pseudintermedius*

Staphylococcus pseudintermedius is a Gram-positive spherically shaped bacterium belonging to the genus *Staphylococcus*. It is a facultatively anaerobic bacterium that is non-motile and does not form spores. Morphologically, *S. pseudintermedius* typically appears as grape-like clusters, although it may also occur as a single cells or paired cocci [29]. *S. pseudintermedius*, closely related to *S. intermedius*, has been identified as a distinct species in 2005 through 16S rRNA gene sequence analysis [5].

S. pseudintermedius has traditionally been identified based on colony morphology, standard phenotypic tests, and distinctive biochemical characteristics, such as positive arginine dihydrolase activity and acid production from β-gentiobiose and d-mannitol, which help differentiate it from other coagulase-positive staphylococci [40]. The colonies of *S. pseudintermedius* are medium-sized, raised, unpigmented, and exhibit characteristic haemolysis patterns, such as incomplete β-haemolysis or complete δ-haemolysis, which may occur individually or in combination (double haemolysis) on sheep or bovine blood agar [40]. However, accurate phenotypic identification has become increasingly challenging due to recent taxonomic revisions within the species. Compounding this difficulty is the identification of *Staphylococcus schleiferi* subsp. *coagulans*, a pathogenic species now recognized for its role in canine

infections, particularly otitis[5]. Alternatively, identification can be achieved using a multiplex-PCR assay targeting the *nuc* gene which encodes a specific nuclease. This opportunistic pathogen is responsible for various infections, including skin diseases such as pyoderma, otitis externa, wound infections, and abscesses, as well as infections in other tissues and cavities [41]. *S. pseudintermedius* is a natural commensal of the skin and mucous membranes in dogs and represents the most frequently isolated bacterial pathogen from canine clinical samples. This coagulase-positive staphylococcal species is predominantly linked to skin and ear infections but can also cause a wide range of community and hospital acquired infections [5], [42]. *S. pseudintermedius* is a primary cause of pyoderma in dogs and serves as a significant reservoir for antimicrobial resistance genes within the genus [21]. In healthy dogs, *S. pseudintermedius* is a natural component of the cutaneous microflora, colonizing the skin, hair follicles, and coat, as well as mucocutaneous regions such as the nose, mouth and anus [5]. This bacterium accounts for approximately 90% of staphylococci isolated from both healthy canine carriers and dogs with underlying skin conditions [6]. Dogs are also the primary host species for *S. pseudintermedius* infections, although it has been sporadically reported in other animals, such as horses, and occasionally in humans [6]. Notably, a recent case of fatal *S. pseudintermedius* infection was documented in a horse [43]. In humans, the first reported infection linked to *S. pseudintermedius* occurred over two decades ago in a dog bite wound [44]. Since then, sporadic cases have been reported, often associated with close contact with pet dogs. In the last ten years, multidrug-resistant strains have spread worldwide, raising concerns about clones that have acquired the *Staphylococcal Chromosomal Cassette* (*SCCmec*), a genetic element that enables the transfer of the methicillin

resistance gene *mecA [39]*. Methicillin-resistant *Staphylococcus pseudintermedius* (MRSP) has been isolated from various hosts, including dogs, cats, and occasionally humans, highlighting its potential for zoonotic transmission[29]. The emergence and global dissemination of MRSP, resistant to nearly all antimicrobial agents available in veterinary practice, have significantly complicated treatment options. Consequently, there is an urgent need for effective strategies to prevent and control *S. pseudintermedius* infections in dogs [45]. One key difference between *S. aureus* and *S. pseudintermedius* is that *S. pseudintermedius* rarely colonizes humans, even those who are frequently in contact with animals [23]. As a results, it is considered less important as a zoonotic pathogen compared to MRSA. However, several reports have documented the transmission of methicillin-susceptible and methicillin-resistant *S. pseudintermedius* between dogs and humans [6], [23], [29]. For example, study examined the presence of *S. pseudintermedius* in 13 dogs with deep pyoderma, their owners, and 13 people with no regular contact with dogs, highlighting the potential for cross-species transmission [46]. Transmission of *S. pseudintermedius* between dogs and their owners has been increasingly reported. While humans are not permanently colonized by this microorganism, they can become transient carriers through close contact with infected dogs [23].

4.5 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a ubiquitous Gram-negative bacterium of the genus *Pseudomonas*, known for its ability to thrive in diverse environments. This bacterium is an aerobic, non-spore forming rod that can grow on standard media across a wide temperature range (4-41°C). It is motile due to polar flagella and produces various diffusible pigments, including pyoverdine (fluorescent green), pyorubin (red brown),

pyomelanin (brown/black) and pyocyanin (blue/green). These pigment are clearly visible in colonies grown on agar media. Additionally, the bacterium produces an aromatic compound called 2-aminoacetophenone, which give its colonies a distinctive grape-like odor [47]. *P. aeruginosa* also secretes various virulence factors, including toxins and enzymes, which play a significant role in tissue damage and the progression of diseases [48]. One of the most significant virulence factors of *P. aeruginosa* is its ability to form biofilms – complex bacterial communities surrounded by a protective matrix of extracellular polymeric substances [49]. These biofilms can attach to various surfaces, such as medical devices or lung tissue, making it challenging for the host's immune system or antibiotics to clear the infection effectively [50]. *P. aeruginosa* also secretes a range of toxins that enhance its virulence [47]. These include exotoxin A, which inhibits protein synthesis, and pyocyanin, which produces reactive oxygen species that can damage host cells. Additionally, the bacterium secretes elastase, an enzyme that breaks down host tissues and disrupts the immune response [51] [47]Furthermore, *P. aeruginosa* has developed several strategies to evade the host immune system, such as producing pigments that hinder immune cell recognition and altering its lipopolysaccharide structure to avoid detection by immune defenses [52]. The diagnosis of *Pseudomonas aeruginosa* relies on its isolation and laboratory identification. The bacterium grows readily on most standard laboratory media, with blood agar and eosin-methylene blue agar being commonly used for isolation. Identification is based on several key characteristics, including its Gram-negative morphology, inability to ferment lactose, positive oxidase reaction, distinctive fruity odor, and ability to grow at 42°C [53]. Early identification is further facilitated by the fluorescence of *P. aeruginosa* colonies under ultraviolet light, which

can also indicate its presence in wound samples. This bacterium is highly versatile, able to thrive in diverse environments [54]. *Pseudomonas aeruginosa* can infect dogs, particularly those with weakened immune systems or underlying health conditions. Dogs can contract the bacterium through contact with contaminated water, soil, or surfaces [55]. Infections can lead to various symptoms, including skin and ear infections, urinary tract infections, and respiratory issues. *P. aeruginosa* frequently exhibit resistance to many commonly used antibiotics, making it a significant challenge in clinical settings. According to 2019 data, it ranks as the as the sixth-leading pathogen in terms of human deaths attributable to bacterial antimicrobial resistance [56]. This bacterium possesses an extensive repertoire of antibiotic resistance mechanisms, including chromosomal determinants and intricate regulatory pathways that mediate both intrinsic and adaptive resistance. Key mechanisms include low outer membrane permeability, the production of chromosomal *AmpC* β-lactamase, and the expression of genes encoding multidrug resistance efflux pumps [57]. These attributes underscore its capacity to withstand diverse antimicrobial therapies [58]. While many strains of *Pseudomonas aeruginosa* remain susceptible to antibiotics such as gentamicin, tobramycin, colistin, and amikacin, the emergence of resistant variants necessitates routine susceptibility testing [57]. For severe *Pseudomonas* infections, particularly in leukopenic patients, a combination therapy of gentamicin and carbenicillin is commonly employed to enhance treatment efficacy [57]. *Pseudomonas aeruginosa*, similar to its role in humans, acts as an opportunistic pathogen in numerous animal species. It is capable of causing a wide array of infections, including those affecting the ears, eyes, urogenital tract, wounds, respiratory system, and skin. These infections typically arise when normal protective barriers are compromised, making

it an uncommon primary pathogen in healthy individuals. However, once established, *P. aeruginosa* infections can be difficult to treat effectively due to its intrinsic resistance mechanisms and adaptability [54]. In dogs, *Pseudomonas aeruginosa* is most frequently associated with cases of otitis; however, its opportunistic nature enables it to cause a wide range of other infections. While less commonly reported, *P. aeruginosa* can also cause infections in cats [48]. Notably, *P. aeruginosa* has been recognized as one of the most significant antimicrobial-resistant (AMR) pathogens affecting dogs and cats within the European Union, as highlighted in a previous scientific assessment [59]. Moreover, dogs can serve as reservoirs for *P. aeruginosa*, potentially transmitting the bacteria to other animals and humans [23].

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Chapter 5: *In vitro* antibacterial activity of hemp (*Cannabis sativa* L.) extract seed oil against multidrug resistant bacterial pathogens in small animal veterinary dermatology

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Abstract

There is an urgent need for alternative antimicrobial therapies in veterinary small animal dermatology due to the limited therapeutic options available for treatment of infections caused by multidrug-resistant bacteria. This study aimed to evaluate the potential of hemp (*Cannabis sativa* L.) seed oil for topical treatment of localized infections of the skin, such as otitis externa. We determined antimicrobial activity by broth microdilution using a strain collection of bacterial pathogens associated with skin infections, including *Staphylococcus pseudintermedius* (n=120), *Staphylococcus aureus* (n=48), and *Pseudomonas aeruginosa* (n=26). Checkerboard dilution tests were used to assess the interaction of hemp seed oil with two antimicrobials used for management of otitis externa, gentamicin and enrofloxacin, while *in vitro* cytotoxicity was evaluated by the cellular 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay on mouse fibroblast cell line L929. Minimum inhibitory concentrations (MICs) in staphylococci (0.025-2% vol/vol) were markedly lower than in *P. aeruginosa* (>0.4% vol/vol). Within *S. pseudintermedius*, methicillin-resistant strains displayed lower susceptibility compared to susceptible strains. Hemp seed oil showed synergy with gentamicin (Fractional Inhibitory Concentration Index < 0.5), reducing the MIC of gentamicin-resistant *S. pseudintermedius* strains (≥16 µg/ml) below the clinical susceptibility breakpoint (≤4 µg/ml). No changes in cell viability were observed at concentrations below 2% vol/vol. These findings suggest that hemp seed oil could be an effective and safe alternative or adjuvant to conventional antimicrobials for managing otitis externa and other skin focal infections caused by staphylococci, including methicillin-resistant strains.

Keywords: Hemp seed oil; topical treatment; dogs; otitis externa; staphylococci; methicillin resistance.

1. Introduction

The rise of multidrug-resistant (MDR) bacterial pathogens in veterinary small animal dermatology, coupled with the shortage of new antimicrobials, underscores the urgent need for new treatment strategies (Martins et al., 2022). Management of MDR bacterial infections has become particularly challenging in the EU after the recent ban of antibiotics that are not authorized for animal use (Schmerold et al., 2023). The EFSA Panel on Animal Health and Welfare has identified *S. pseudintermedius* and *P. aeruginosa* as the most relevant antimicrobial-resistant bacteria in small animals across the EU (Nielsen et al., 2021). Methicillin-resistant *Staphylococcus pseudintermedius* (MRSP) is a major concern due to the typical MDR profiles of certain epidemic lineages, such as clonal complexes 71 and 45 (Dos Santos et al., 2016).

This bacterial species is the most prevalent cause of canine bacterial infections in dogs, especially pyoderma, otitis externa and wound infection (Bannoehr & Guardabassi, 2012). Among Gram-negative pathogens, *Pseudomonas aeruginosa* is a major contributor to canine otitis externa, exacerbated by the increasing prevalence of MDR strains (De Martino et al., 2016). While dogs are not typically colonized by *Staphylococcus aureus*, skin, post-surgical and wound infections caused by methicillin-resistant strains (MRSA) are sporadically reported, posing significant treatment challenges (Pantosti, 2012; Haag et al., 2019).

As the demand for alternatives to conventional antibiotics grows, the scientific community is placing greater emphasis on the antimicrobial properties of natural substances (Helmy et al., 2023). Plants and their

derivatives have shown considerable antimicrobial potential, with numerous studies demonstrating the *in vitro* efficacy of plant extracts against MRSA (Hammer et al., 1999; Meroni et al., 2020). Among plant-derived substances, hemp and its extracts have attracted significant attention(Nissen et al., 2010). Of the various hemp varieties, *Cannabis sativa* L. is the most extensively studied in medicine due to its adaptability and ease of cultivation in different climates (Montserrat-De La Paz et al., 2014; Mikulcová et al., 2017). *Cannabis sativa* L. has demonstrated multiple pharmacological properties, including antibacterial activity against MRSA and MRSP, which is attributed to the presence of cannabinoids (Chiong et al., 2024).

Hemp extracts contain a broad range of bioactive compounds that act synergistically, making it more difficult for bacteria to develop resistance (Martinenghi et al., 2020; Luz-Veiga et al., 2023).

The antimicrobial effects of hemp extracts are often attributed to the presence of cannabinoids like THC and CBD (Appendino et al., 2008). Although the exact mechanism behind the antimicrobial action of hemp extracts remains unclear, a study suggests that cannabidiol may be associated with the rapid disruption of bacterial cytoplasmic membranes, though it is uncertain whether this effect involves a specific molecular target (Blaskovich et al., 2021). Unlike hemp extracts such as essential oils, hemp seed oil, obtained by cold pressing hemp seeds, is free of cannabinoids, including THC and CBD. Any detectable traces of these compounds in products like the oil are probably due to accidental contamination from the plant's floral part (Montserrat-De La Paz et al., 2014; Mikulcová et al., 2017). It is interesting to investigate whether the hemp seed oil could still exert an antimicrobial effect, as it might reveal other potential bactericidal molecules.

Topical therapy with cannabidiol is a promising option in dermatology due to its effectiveness against biofilms, low propensity to induce resistance, and demonstrated *in vivo* efficacy (Blaskovich et al., 2021). Additionally, topical cannabinoids have shown beneficial effects on the skin, including anti-inflammatory, anti-itching, analgesics, wound healing and anti-proliferative properties (Filipiuc et al., 2023). In this study, we investigated the potential of hemp extract seed oil in veterinary dermatology by assessing its antibacterial activity against MDR strains of bacterial pathogens associated with skin infections in dogs. We also explored the synergistic effect of hemp seed oil when combined with gentamicin and enrofloxacin, two antimicrobial agents used in veterinary practice for topical treatment of otitis externa.

2. Materials and Methods

2.1 Bacterial strains

A total of 198 bacterial strains, including 124 clinical *S. pseudintermedius* isolates (70 MRSP and 54 MSSP), 48 clinical *S. aureus* isolates (19 MRSA and 29 MSSA) and 26 *P. aeruginosa* isolates, collected from dogs between 2008 and 2023 as part of routine diagnostics at the Department of Veterinary Medicine at the University of Copenhagen (Denmark) and at the Department of Veterinary Medicine at the University of Perugia (Italy) were included in this study (Table S1, see Supplementary Materials).

2.2 Hemp extract seed oil

The hemp extract seed oil was provided by BioAgrigea Company (Padova, Italy) and was obtained by the cold extraction method from the hemp variety FUTURA 75. Cannabinoid titration was performed by ultra-high performance liquid chromatography-MS/MS (UHPLC-MS/MS) with an analyte limit of detection of 0.01 mg/ml. Prior to testing, the oil was diluted in DMSO to a maximum concentration of 6.4% vol/vol.

2.3 MIC testing

Hemp seed oil minimal inhibitory concentration (MIC) was determined in the range 0.4-0.007% vol/vol via the broth microdilution method according to CLSI guidelines (Clinical and Laboratory Standards Institute (CLSI), 2018). *S. aureus* ATCC 29213 and *P. aeruginosa* ATCC 27853 were used as quality control strains. The effect of high concentration of DMSO on bacterial growth was assessed by growth kinetics on representative strains. Statistical analysis was performed to assess the significance of differences between groups. Results were interpreted using the Student's T test to compare means between methicillin-resistant and susceptible strains groups. A p value of ≤ 0.05 was considered statistically significant. All statistical analyses were conducted using GraphPad Prism version 8 (GraphPad Software, San Diego, USA).

2.4 Checkerboard assay

After testing the gentamicin and enrofloxacin MICs by broth microdilution (range 128-0.25 µg/mL), checkerboard assays were performed to understand interactions of these antimicrobials with hemp extract seed oil as previously described (Sopirala et al., 2010) with some modifications. Briefly, antimicrobials were serially diluted 2-fold in the rows in a 96-well microtiter plate, while the hemp seed oil was serially diluted 2-fold in the columns to create a matrix in which each well contained a combination of both agents at different concentrations. Fifty microliters of the bacterial suspension were inoculated into each well at a final concentration of 5 x 10^5 CFU/ml. The plates were incubated aerobically at 37 °C for 20 hours. After reading the optical turbidity of the wells, the Fractional Inhibitory Concentration Index (FICI) was calculated according to the following formula: $FICI = [MIC_{A(A+B)}/MIC_A + MIC_{B(A+B)}/MIC_B]$, where $MIC_{A(A+B)}$ and $MICB_{(A+B)}$ represent the concentrations of compounds A and B,

respectively, in the combination, while MIC_A and MIC_B represent the MIC of each compound individually. The interaction of the two compounds was interpreted as synergy, antagonism or indifference for FICI values of ≤ 0.5, > 4.0 and > 0.5 to 4.0, respectively.

2.5 Cell cytotoxicity assay

MTT test was performed on L929 cell line exposed to different concentrations of hemp extract seed oil (2%, 1%, 0.5%, 0.25%, 0.125% v/v) or DMSO, which was tested as the same concentration as vehicle control. The L929 cell line was cultivated in low glucose DMEM supplemented with 10% FBS, penicillin (100 IU/ml) and streptomycin (100 µg/ml) at 37°C and 5% CO_2. L929 were seeded in 96-well plates at 10^4 cells/cm^2 and incubated for 24 hours. The medium was then replaced with 100 µl of complete cell medium supplemented hemp seed oil or DMSO. After 24 hours, 10 µl of MTT 5 mg/ml were added to each well and incubated for 4 hours. At the end of the incubation, the cell medium was removed, and the formazan precipitates was solubilized with 100 µl DMSO and the absorbance was measured at 570 and 630 nm. The relative cell viability was calculated by comparing the absorbance value of the treated cells with that of the untreated cells. The analysis was performed in three independent experiments and each dilution was performed in triplicate, and results were compared using one-way analysis of variance (ANOVA) with post-hoc Tukey's HSD and Bonferroni correction in GraphPad Prism version 8 (GraphPad Software, San Diego, USA). *P* values ≤ 0.05 were considered significant.

3. Results

3.1 Hemp seed oil cannabinoid concentrations

Prior to testing, the cannabinoid concentration of the hemp extract seed oil was determined by UHPLC-MS/MS. Cannabidiolic acid showed the highest concentration (1.3 mg/ml), followed by CBD (0.29 mg/ml) and delta-9-tetrahydrocannabinolic acid (0.04 mg/ml). Cannabinol and Δ^9-THC concentrations were under the limit of detection (< 0.01 mg/ml).

3.2 Antimicrobial activity of hemp seed oil

Growth kinetics analysis of representative bacteria strains in the presence of DMSO showed that concentrations below 1.6 % had no effect on the growth dynamics of the bacteria compared to the untreated control (Fig. S1, see Supplementary Materials). This concentration was selected as the highest concentration tested in MIC assay. MIC values of hemp seed oil on 124 clinical *S. pseudintermedius* strains ranged between 0.025 to >0.4% (vol/vol) (Fig. 1A). MRSP strains generally displayed higher MIC values (above 0.1%) compared to susceptible strains (Fig 1A), with a significant difference observed between MRSP and MSSP (Fig. 1C; $p < 0.001$). In contrast, no significant difference was noted between methicillin-resistant and susceptible *S. aureus* strains (Fig. 1D). All *P. aeruginosa* strains displayed MIC values greater than 0.4% (vol/vol).

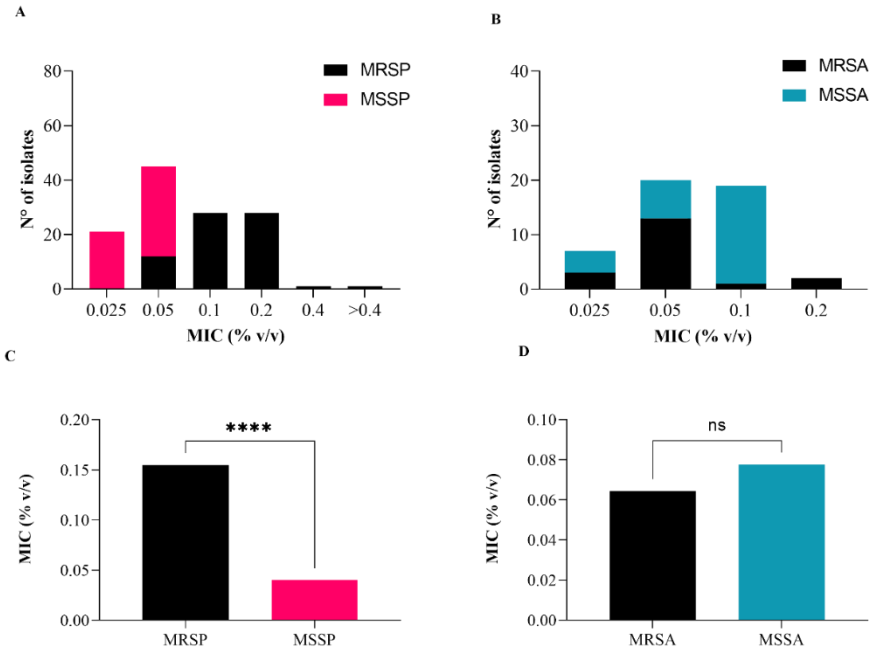

Fig. 1. Distribution of Minimum Inhibitory Concentration (MIC) values (% v/v) of hemp seed oil for *Staphylococcus* isolates. (A) MIC distribution for methicillin-resistant *Staphylococcus pseudintermedius* (MRSP) and methicillin-susceptible *Staphylococcus pseudintermedius* (MSSP), showing higher susceptibility of MSSP isolates at lower MIC values. (B) MIC distribution for methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-susceptible *Staphylococcus aureus* (MSSA), with MSSA isolates demonstrating greater sensitivity to lower concentrations of hemp oil. (C) Results show a statistically significant difference ($p < 0.001$) in MIC values between MRSP and MSSP isolates, indicating that MSSP strains are more susceptible to hemp seed oil. (D) No statistically significant difference in MIC values is observed between MRSA and MSSA isolates, suggesting similar levels of susceptibility to hemp seed oil between these two groups.

3.3 Synergistic activity of hemp seed oil in combination with gentamicin

Thirteen gentamicin-resistant MRSP strains were selected for checkerboard assay. These exhibited a gentamicin MIC of 64-128 µg/mL (Table 1). The hemp seed oil showed strong synergistic interactions (FICI < 0.5) with gentamicin for all isolates tested with at least 4-fold decrease of gentamicin MIC (Table 1).

The hemp seed oil did not show any synergistic interactions (0, 5 > FICI > 4.0) with enrofloxacin for all MRSP strains tested (n=13) and with both gentamicin and enrofloxacin for the *P. aeruginosa* strains tested (n=7). Hemp sensitized gentamicin-resistant MRSP strains (R≥16 µg/ml) to MIC below the CLSI susceptibility breakpoint (S≤4 µg/ml).

Table 1. Checkerboard results for gentamicin and hemp seed oil combination in 13 clinical MRSP strains.

Isolate ID	**MIC_A[a]**	**$MIC_{A(A+B)}$[b]**	**MIC_B[c]**	**$MIC_{B(A+B)}$[d]**	**FICI[e]**
37711-3	128	1	0,1	0,05	0,25
45250-1	64	2	0,1	0,05	0,25
45250-2	64	4	0,05	0,025	0,375
46507	64	4	0,05	0,025	0,375
41484	128	2	0,2	0,1	0,187
42654	128	2	0,1	0,05	0,188
27150	64	4	0,1	0,05	0,137
32908	64	2	0,1	0,05	0,137
31369	64	2	0,1	0,05	0,137
30655	64	1	0,1	0,05	0,075
30538	64	1	0,2	0,05	0,131
30511	64	1	0,2	0,05	0,131

28522	64	2	0,2	0,025	0,069

[a]MIC_A, MIC () of gentamicin alone.

[b]$MIC_{A(A+B)}$, MIC () of gentamicin in combination with Hemp seed oil.

[c]MIC_B, MIC (v/v) of Hemp seed oil alone.

[d]$MIC_{B(A+B)}$, MIC () of Hemp seed oil in combination with gentamicin.

[e]FICI is given for the combination with the highest degree of synergy.

3.3 Effect of hemp seed oil on L929 cell line

The cellular 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay MTT assay was performed on mouse fibroblast L929 cells to evaluate the cytotoxicity effect of the hemp extract seed oil. Incubation of cells with hemp seed oil concentrations of 1% vol/vol or below did not lead to changes in cell viability (Fig. 2). However, at 2% vol/vol of hemp seed oil a marked decrease in cell viability was observed (relative viability 4%). DMSO did not show any reduction in cell viability at any of the tested concentrations (Fig. 2).

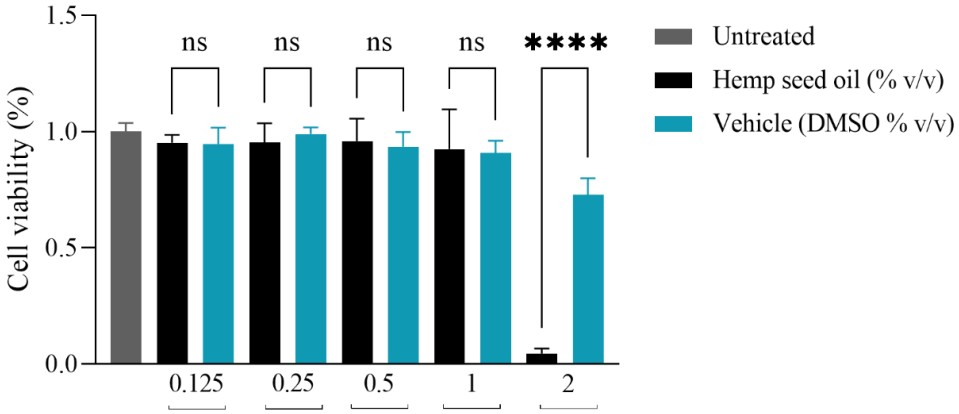

Fig. 2 Relative viability of L929 cell line treated with increasing concentrations of hemp seed oil, as compared to the vehicle control (DMSO), after 24h of exposure, measured by MTT assay. No statistically significant impact on cell

viability was observed for both hemp seed oil and DMSO at concentrations ≤ 1%. A significant decrease in cell viability ($p < 0.001$) was detected at 2% vol/vol between hemp seed oil and vehicle control. Data are presented as mean ± SD of three independent experiments, each comprising three replicates.

4. Discussion

We investigated the antimicrobial activity of hemp extract seed oil against pathogenic bacteria in small animal veterinary dermatology. To date, the focus has primarily been on the essential oil of hemp or on specific cannabinoids such as CBD and THC (Nissen et al., 2010; Puškárová et al., 2017; Iseppi et al., 2019; Nocera et al., 2020). To the best of our knowledge, this is the first study to evaluate the antimicrobial activity of hemp extract seed oil against MDR pathogens isolated from dogs using a quantitative approach. In all previous studies on hemp seed oil, the antimicrobial effect was assessed using semi-quantitative methods, such as disk diffusion, targeting the bacteria primarily responsible for spoilage, including gram-positive *Bacillus subtilis*, *Bacillus cereus*, *Enterococcus faecalis* and *Staphylococcus aureus* and the Gram-negative *Citrobacter freundii*, *Escherichia coli*, *Proteus vulgaris*, *Pseudomonas aeruginosa* and *Serratia marcescens* (Nissen et al., 2010; Mikulcová et al., 2017;). To allow a quantitative approach, we prepared scalar dilutions of the seed oil dissolved by diluted DMSO, which is widely used to solubilize oils without interfering with their biological effects when used at subinhibitory concentrations (Modrzyński et al., 2019; Tunçer & Gurbanov, 2023). The highest seed oil concentration that could be solubilized was 0.4% with 1.6% DMSO, and we demonstrated that this concentration does not interfere with bacterial growth.

Our results indicate that hemp seed oil can exert strong antimicrobial activity against staphylococci, including MRSP and MRSA (Fig. 1). Direct comparison with existing literature is not feasible, as no similar studies have been reported. Additionally, comparing the antimicrobial activity of hemp extracts from different studies can be problematic and misleading due to variations in evaluation methods and various factors that influence the chemical composition of these extracts, such as the plant organ used for extraction (e.g. leaves, flowers or seeds), as well as plant's maturity and harvesting stage. Interestingly, MRSP strains displayed higher MICs compared to susceptible strains, a trend not observed for MRSA (Fig. 1). This suggests that the structural or functional changes in the cell wall associated with methicillin resistance in *S. pseudintermedius*, but not in *S. aureus*, may interfere with penetration and/or activity of hemp seed oil.

While investigating the reason behind this result goes beyond the scope of this study, further research is warranted to elucidate the potential relationships between methicillin resistance and susceptibility to hemp seed oil.

It was not surprising that hemp seed oil showed lower antimicrobial activity against *P. aeruginosa* since no studies on seed oil or essential oils, including those from hemp, have demonstrated a good efficacy against this Gram-negative species. According to De Sousa et al. (2023), this could be due to the presence of liposaccharides in Gram-negative bacteria, which prevent the attachment of seed oil to the cell membrane.

As widely reported in the literature, the antimicrobial properties of hemp extracts are related to the presence of cannabinoids such as THC and CBD (Baswan et al., 2020; Martinenghi et al., 2020; Blaskovich et al., 2021; Robaina Cabrera et al., 2021; Luz-Veiga et al., 2023). It should be noted that the hemp seed oil used in this study was THC-free and had a very low

CBD content (0.285 mg/ml), detectable only as an impurity during the extraction processes. These levels are too low to exhibit antibacterial activity. Hemp seed oil has a relatively complex macro composition, and therefore, numerous compounds beyond cannabinoids, whether alone or in combination, have the potential to exert antimicrobial activity (Leizer et al., 2000).

In current veterinary dermatology, topical treatment is recommended over systemic treatment for managing otitis externa and localized skin infections because it reduces the risk of side effects and lowers the likelihood of selecting resistant strains at other body sites, such as the intestinal tract, where most bacteria reside (Bajwa, 2019). Here, we demonstrated a synergistic effect of hemp seed oil with gentamicin, resulting in a significant reduction in the MIC below the CLSI clinical susceptibility breakpoint (S ≤4 µg/ml) in *S. pseudintermedius*. While the mechanism underlying this synergy was not investigated, this result suggests that hemp seed oil may be a viable option for managing localized skin and ear infections caused by gentamicin-resistant strains, either alone or in combination with gentamicin. By potentiating the activity of gentamicin, hemp seed oil could also enhance efficacy in treating otitis externa caused by gentamicin-susceptible strains, as the high amounts of exudate in the ear canal are likely to interfere with stability and activity of the antibiotic (Carlotti, 1991; Bajwa, 2019).

To support the future application of hemp seed oil as a topical treatment, the cytotoxic effect was evaluated on the fibroblast cell line L929, which was chosen because it is widely used for testing the cytotoxicity of natural products. As demonstrated by the MTT assay results, hemp extract seed oil at concentrations corresponding to the MIC values recorded for the bacterial strains included in the study does not produce any toxic effects

on the cellular growth, indicating that it is safe for use in topical applications.

5. Conclusions

Our work demonstrates the antibacterial activity of hemp seed oil against staphylococci associated with skin infections in dogs at concentrations that are not cytotoxic. The antimicrobial activity against MRSP and MRSA, along with the synergistic effect observed when combined with gentamicin, underscores the potential of hemp seed oil as an alternative or adjunctive topical antimicrobial therapy for otitis externa and other focal infections in small animal veterinary dermatology.

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Credit authorship contribution statement

Conceptualization: VT, MP, ER, GS; Data curation: VT, ER; Investigation: VT, MP, LM; Methodology: VT, MP, GS, LG; Resources: MDB; Writing – original draft: VT, LG, PCP; Writing – review and editing: ADS, GdR, MP, LG, PCP.

Declaration of Competing Interest

VT, MP, ER, GS, LM, GdR, ADS, LG and PCP declare no competing interest concerning the research, authorship, publication of this article and/or financial and personal relationship that could inappropriately

influence this work. MDB is the CEO of BioAgrigea Company who provided the test compound hemp seed oil.

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Supplementary Materials

TABLE S1. Strains used in this study.

Species	Isolate ID	Country	Year	Source	Methicillin-resistance
Staphylococcus pseudintermedius	23639	Denmark	2008	Ear	MRSP
S. pseudintermedius	25561-1	Denmark	2009	Nose	MRSP
S. pseudintermedius	26012	Denmark	2009	Skin	MRSP
S. pseudintermedius	26071-1	Denmark	2009	Skin	MRSP
S. pseudintermedius	26461	Denmark	2010	Wound	MRSP
S. pseudintermedius	26893	Denmark	2010	Ear	MRSP
S. pseudintermedius	27150	Denmark	2010	Swab	MRSP
S. pseudintermedius	27114	Denmark	2010	Wound	MSSP
S. pseudintermedius	27153-2	Denmark	2010	Ear	MSSP
S. pseudintermedius	27242-1	Denmark	2010	Bone	MSSP
S. pseudintermedius	27256	Denmark	2010	Skin	MSSP
S. pseudintermedius	27258	Denmark	2010	Bone	MSSP
S. pseudintermedius	27272	Denmark	2010	Wound	MSSP
S. pseudintermedius	27282-1	Denmark	2010	Urine	MSSP
S. pseudintermedius	27311	Denmark	2010	Ear	MSSP
S. pseudintermedius	28522	Denmark	2011	Skin	MRSP
S. pseudintermedius	27364	Denmark	2011	Wound	MSSP
S. pseudintermedius	27372-1	Denmark	2011	Ear	MSSP
S. pseudintermedius	27382	Denmark	2011	Ear	MSSP
S. pseudintermedius	27391	Denmark	2011	Skin	MSSP
S. pseudintermedius	27407	Denmark	2011	Urine	MSSP
S. pseudintermedius	27425	Denmark	2011	Ear	MSSP
S. pseudintermedius	27427	Denmark	2011	Skin	MSSP
S. pseudintermedius	27440	Denmark	2011	Urine	MSSP
S. pseudintermedius	27468	Denmark	2011	Ear	MSSP
S. pseudintermedius	27470-1	Denmark	2011	Ear	MSSP
S. pseudintermedius	27472	Denmark	2011	Wound	MSSP
S. pseudintermedius	27475	Denmark	2011	Ear	MSSP
S. pseudintermedius	27476	Denmark	2011	Skin	MSSP
S. pseudintermedius	27519	Denmark	2011	Swab	MSSP
S. pseudintermedius	27523-1	Denmark	2011	Ear	MSSP
S. pseudintermedius	27539	Denmark	2011	Skin	MSSP
S. pseudintermedius	27540-1	Denmark	2011	Ear	MSSP
S. pseudintermedius	27548	Denmark	2011	Skin	MSSP
S. pseudintermedius	27556	Denmark	2011	Skin	MSSP
S. pseudintermedius	27557	Denmark	2011	Skin	MSSP
S. pseudintermedius	27607	Denmark	2011	Skin	MSSP
S. pseudintermedius	28819	Denmark	2012	Skin	MRSP

S. pseudintermedius	29237-1	Denmark	2012	Ear	MRSP
S. pseudintermedius	30058	Denmark	2012	Ear	MRSP
S. pseudintermedius	30179	Denmark	2012	Wound	MRSP
S. pseudintermedius	30511	Denmark	2012	Skin	MRSP
S. pseudintermedius	30538	Denmark	2012	ND	MRSP
S. pseudintermedius	30565-1	Denmark	2012	Skin	MSSP
S. pseudintermedius	30655	Denmark	2013	Skin	MRSP
S. pseudintermedius	30755	Denmark	2013	Skin	MRSP
S. pseudintermedius	31304	Denmark	2013	Nose	MRSP
S. pseudintermedius	31369	Denmark	2013	ND	MRSP
S. pseudintermedius	31413	Denmark	2013	Joint fluid	MRSP
S. pseudintermedius	31473	Denmark	2013	Wound	MSSP
S. pseudintermedius	31600	Denmark	2013	Skin	MRSP
S. pseudintermedius	32908	Denmark	2014	Vagina	MRSP
S. pseudintermedius	33228	Denmark	2014	Skin	MRSP
S. pseudintermedius	33231-2	Denmark	2014	Nose	MRSP
S. pseudintermedius	33238	Denmark	2014	Wound	MRSP
S. pseudintermedius	33648	Denmark	2014	Skin	MRSP
S. pseudintermedius	37028	Denmark	2016	Skin	MRSP
S. pseudintermedius	37037	Denmark	2016	Wound	MRSP
S. pseudintermedius	37235	Denmark	2016	Skin	MRSP
S. pseudintermedius	37261	Denmark	2016	Skin	MRSP
S. pseudintermedius	37526-1	Denmark	2016	Skin	MRSP
S. pseudintermedius	37242	Denmark	2016	Furuncle	MSSP
S. pseudintermedius	37264	Denmark	2016	Urine	MSSP
S. pseudintermedius	37611	Denmark	2016	Wound	MSSP
S. pseudintermedius	38637	Denmark	2017	Wound	MRSP
S. pseudintermedius	39490	Denmark	2017	Wound	MRSP
S. pseudintermedius	41258	Denmark	2018	Wound	MRSP
S. pseudintermedius	41470	Denmark	2018	Wound	MRSP
S. pseudintermedius	42654	Denmark	2019	Skin	MRSP
S. pseudintermedius	44704	Denmark	2020	Wound	MRSP
S. pseudintermedius	45201	Denmark	2020	Wound	MRSP
S. pseudintermedius	45250-2	Denmark	2020	Ear	MRSP
S. pseudintermedius	46507	Denmark	2021	Wound	MRSP
S. pseudintermedius	46552-2	Denmark	2021	Skin	MRSP
S. pseudintermedius	46464-1	Denmark	2021	Skin	MRSP
S. pseudintermedius	11385	Italy	2015	Wound	MRSP
S. pseudintermedius	14409	Italy	2019	Skin	MRSP
S. pseudintermedius	14616	Italy	2019	Skin	MRSP
S. pseudintermedius	15049	Italy	2019	Skin	MRSP
S. pseudintermedius	15120	Italy	2019	Skin	MRSP
S. pseudintermedius	15397	Italy	2020	Nodule	MRSP
S. pseudintermedius	17607	Italy	2022	Ear	MRSP
S. pseudintermedius	17612	Italy	2022	Skin	MRSP
S. pseudintermedius	17679	Italy	2022	Ear	MRSP

S. pseudintermedius	17687	Italy	2022	Ear	MRSP
Staphylococcus aureus	25809	Denmark	2009	Wound	MSSA
S. aureus	25855	Denmark	2009	Trachea	MSSA
S. aureus	26246	Denmark	2010	Wound	MSSA
S. aureus	26252	Denmark	2010	Wound	MSSA
S. aureus	26466	Denmark	2010	Wound	MSSA
S. aureus	26604	Denmark	2010	Wound	MSSA
S. aureus	26735	Denmark	2010	Wound	MSSA
S. aureus	26926	Denmark	2010	Joint	MSSA
S. aureus	26966	Denmark	2010	Swab	MSSA
S. aureus	27266	Denmark	2010	Skin	MSSA
S. aureus	27474	Denmark	2011	Synovia	MSSA
S. aureus	27479	Denmark	2011	ND	MSSA
S. aureus	27871	Denmark	2011	Trachea	MSSA
S. aureus	27915	Denmark	2011	Wound	MSSA
S. aureus	27915-1	Denmark	2011	Wound	MSSA
S. aureus	28264	Denmark	2011	Wound	MSSA
S. aureus	28280-1	Denmark	2011	Tumor	MSSA
S. aureus	30045	Denmark	2012	Wound	MSSA
S. aureus	46013	Denmark	2021	Wound	MSSA
S. aureus	46026	Denmark	2021	Wound	MSSA
S. aureus	46129	Denmark	2021	Wound	MSSA
S. aureus	46198	Denmark	2021	Wound	MSSA
S. aureus	47671	Denmark	2022	Wound	MSSA
S. aureus	47987	Denmark	2022	Nose	MSSA
S. aureus	47994	Denmark	2022	Skin	MSSA
S. aureus	48297	Denmark	2022	Wound	MSSA
S. aureus	48346	Denmark	2022	Urine	MSSA
S. aureus	48597	Denmark	2022	Wound	MSSA
S. aureus	49413	Denmark	2023	Wound	MSSA
S. aureus	32347	United Kingdom	2019	ND	MRSA
S. aureus	32420	United Kingdom	2019	ND	MRSA
S. aureus	32760	United Kingdom	2019	ND	MRSA
S. aureus	32761	United Kingdom	2019	ND	MRSA
S. aureus	32775	United Kingdom	2019	ND	MRSA
S. aureus	32786	United Kingdom	2019	ND	MRSA
S. aureus	32869	United Kingdom	2019	ND	MRSA
S. aureus	33876	United Kingdom	2019	ND	MRSA
S. aureus	33942	United Kingdom	2019	ND	MRSA
S. aureus	36844	United Kingdom	2019	ND	MRSA
S. aureus	28217	United Kingdom	2019	ND	MRSA

S. aureus	30259	United Kingdom	2019	ND	MRSA
S. aureus	32505	United Kingdom	2019	ND	MRSA
S. aureus	32508	United Kingdom	2019	ND	MRSA
S. aureus	32533	United Kingdom	2019	ND	MRSA
Pseudomonas aeruginosa	48133-2	Denmark	2022	Ear	-
P. aeruginosa	48161	Denmark	2022	Ear	-
P. aeruginosa	48246	Denmark	2022	Ear	-
P. aeruginosa	48294	Denmark	2022	Ear	-
P. aeruginosa	48285	Denmark	2022	Ear	-
P. aeruginosa	48313-1	Denmark	2022	Ear	-
P. aeruginosa	48332-1	Denmark	2022	Ear	-
P. aeruginosa	48340	Denmark	2022	Ear	-
P. aeruginosa	48353	Denmark	2022	Ear	-
P. aeruginosa	48354	Denmark	2022	Ear	-
P. aeruginosa	48409	Denmark	2022	Ear	-
P. aeruginosa	48410-1	Denmark	2022	Ear	-
P. aeruginosa	48410-2	Denmark	2022	Ear	-
P. aeruginosa	48422-1	Denmark	2022	Ear	-
P. aeruginosa	49679	Denmark	2023	Wound	-
P. aeruginosa	14710	Italy	2019	Ear	-
P. aeruginosa	15250	Italy	2019	Ear	-
P. aeruginosa	15255	Italy	2019	Ear	-
P. aeruginosa	15269	Italy	2019	Ear	-
P. aeruginosa	15542	Italy	2020	Ear	-
P. aeruginosa	15844	Italy	2020	Ear	-
P. aeruginosa	16051	Italy	2020	Ear	-
P. aeruginosa	16077	Italy	2020	Ear	-
P. aeruginosa	16078	Italy	2020	Ear	-
P. aeruginosa	16079	Italy	2020	Ear	-
P. aeruginosa	16105	Italy	2020	Ear	-

ND, not determined

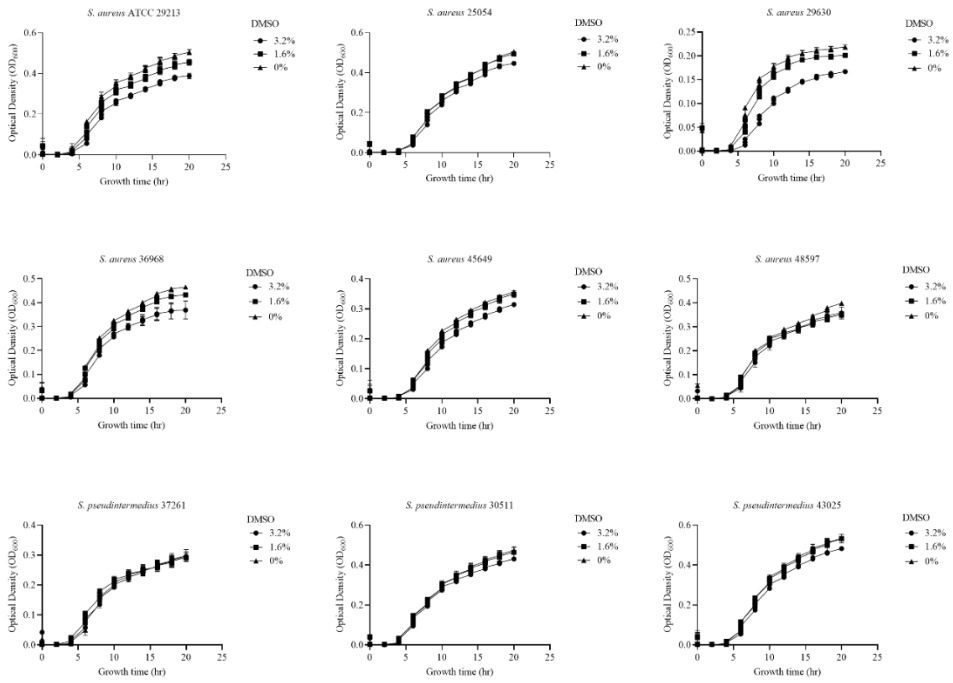

Figure S1. Effect of DMSO the growth of staphylococcal strains. Strains were grown in MHB supplemented or not with DMSO at 3.2% and 1.6%. Growth (OD_{600}) was monitored periodically for up to 24 hours.

Chapter 6: Wound healing effect of Hemp (*Cannabis sativa L.*) seed oil

1. Introduction

Wound healing is a multifaceted biological process essential for the restoration of tissue integrity following injury. It is driven by a tightly regulated series of cellular and biochemical events that work in concert to repair and regenerate damaged tissues [1]. This intricate process involves dynamic interactions among local cells, the vasculature, soluble mediators, and the extracellular matrix, progressing through overlapping yet interdependent phases: inflammation, tissue repair, and maturation [2]. Key mechanisms such as wound contraction, re-epithelialization, tissue remodeling, and granulation tissue formation, supported by angiogenesis, are critical to achieving successful tissue regeneration [3]. Cytokines and growth factors play pivotal roles in orchestrating these processes, initiating and sustaining the repair cascade. However, not all wounds progress seamlessly through these phases, which can result in chronic or non-healing wounds [4]. This underscores the importance of understanding the mechanisms underlying wound healing to develop targeted strategies for intervention. In human medicine, wound healing represents a global healthcare challenge, with an estimated 1–1.5% of the population experiencing severe wound-related complications [5]. Similarly, in veterinary practice, wound management is a common clinical concern in both general and specialized settings. While it is generally assumed that wound healing in domestic animals such as dogs and cats follows similar pathways and involves comparable mediators as in humans, specific research in these species remains limited [6]. A deeper understanding of the cellular and molecular processes governing wound healing across species is crucial for optimizing therapeutic approaches. In this scenario,

the development of innovative wound treatments that are effective, safe, and economically sustainable is of critical importance. Natural products, characterized by their diverse bioactive compounds, represent a promising alternative strategies in wound management [7]. Numerous plants have been extensively investigated for their wound-healing properties in various *in vitro* and *in vivo* models. Among these, particular attention has been given to *Aloe vera* [8], *Curcuma longa*[9], *Calendula officinalis* [10], and *Camellia sinensis* [11], due to their well-documented bioactive compounds and therapeutic potential. Notably, *Cannabis sativa* has garnered significant scientific interest for its wound-healing capabilities. *Cannabis sativa L.*, commonly known as hemp, is an annual plant from the *Cannabaceae* family that grows in diverse environmental conditions [12]. Numerous scientific evidences support the therapeutic potential of cannabis, demonstrating a broad spectrum of pharmacological effects, including anti-inflammatory [13], antidiabetic [14], neuroprotective [15], anticancer [16], antioxidant [17], antimicrobial [18], antiviral [19], and antifungal properties [20]. Due to its diverse biological activities, *C. sativa* is utilized in the treatment of various conditions, such as skin disorders, cancer, neurodegenerative diseases like Alzheimer's and Parkinson's, epilepsy, and post-traumatic stress disorder [7]. The therapeutic effects of cannabis are modulated by a range of phytochemicals, which vary in their concentration, stability, volatility, pharmacological activity, physicochemical properties, and synergistic interactions [21]. Hemp seed oil, derived from the seeds of *Cannabis sativa L.*, is recognized for its nutritional, bioactive, and health-promoting properties [22]. However, the specific wound healing activity of hemp seed oil remains uncertain, with no data available. The aim of this preliminary study is to evaluated the

wound healing ability of hemp seed oil for the potential future treatment of skin wounds in veterinary medicine.

2. Materials and Methods

2.1 Hemp extract seed oil

The hemp extract seed oil was provided by the BioAgrigea Company (Padova, Italy) and its cannabinoid composition is detailed in table 1. The seed oil was obtained through the cold-press extraction method from the *FUTURA 75* hemp variety.

Sample	Analyte	Concentration (mg/mL)
Cold-pressed Hemp (*Cannabis sativa*) seed oil	CBD	0,285
	Δ9-THC	N.D
	CBN	N.D
	CBDA	1,300
	Δ9-THCA	0,040

Detection limit on UHPLC-MS/MS system 0,01 mg/mL
N.D: value below detection limit
CBD: cannabidiol
Δ9-THC: Delta-9-Tetrahydrocannabinol
CBN: Cannabinol
CBDA: Cannabidiolic acid
Δ9-THCA: Delta-9-Tetrahydrocannabinolic acid

Table 1: Cannabinoid titration

2.2 Scratch wound healing assay

A wound healing assay was performed to measure the effect of the hemp seed oil on L929 cell line. The L929 cells were seeded at 2x10^4 cells/cm^2 into 48-well plates and incubated for 24 hours. The cell monolayer was manually scratched with a 200 µl plastic tip and treated with DMEM supplemented with 0,25 and 0,50% hemp seed oil, only

DMEM was used as a negative control, DMEM supplemented with 2% of FBS was used as a positive control (data not show). Prior to testing, the oil was diluted in DMSO to a maximum concentration of 6.4% vol/vol. Each condition was performed in triplicate. Photos of wound areas were taken at 0, 24 and 48 hours with an inverted microscope equipped with a digital camera. Photos were analyzed with "Wound healing size tool update" plugin on ImageJ Fiji [23]. The recovery was calculated as a percentage as the ratio between the analysed sample and the area at the time 0. Statistical tests were considered as significance at the level of 5% ($p < 0.05$).

2.3 Statistical analysis

Scratch assay data are presented as mean ± standard deviation (SD). Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by post-hoc Tukey's HSD and Bonferroni tests to determine differences between groups. All analysis were conducted using GraphPad Prism version (GraphPad Software, San Diego, USA). A significance level of $p \leq 0.05$ was considered for all statistical tests.

3. Results

3.1 Scratch wound healing assay

Results reported in figure 1 showed scratch wounds in L929 cells at 0 h, with wound status shown at 24 and 48 hours post-scratch, following treatment with DMEM alone, vehicle control (DMSO), hemp seed oil concentrations and FBS 2% (Fig 1). FBS was added as a positive control to ensure that, when exposed to a wound healing enhancement stimulus, the L929 cells would close the wound within 48 hours, as expected.

Specifically, the treatment with hemp seed oil 0.25% exhibits comparable wound healing capacity to the vehicle (DMSO) and DMEM only, with no

substantial enhancement observed overall. On the contrary, at the highest concentration of hemp seed oil tested (0.5% v/v), the effect is lower than the baseline condition measured at time 0. (Fig. 1).

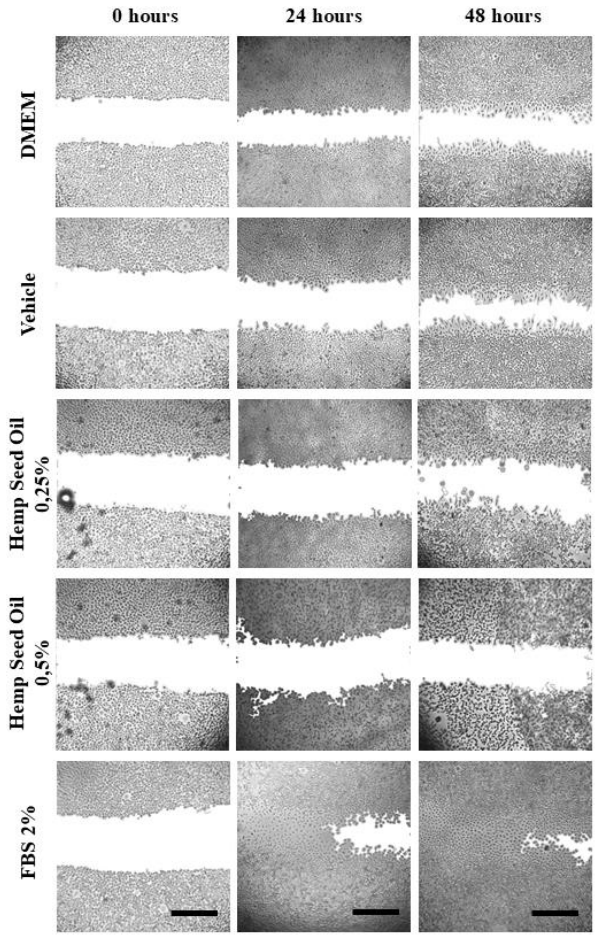

Fig. 1 Representative images of wound healing assay with seed oil treatments at different concentrations (scale bar 500 μm).

No statistically significant difference was observed between vehicle (DMSO) and hemp seed oil 0.25% (v/v) at the assessed time points. Conversely, a statistically significant difference was observed between the

vehicle (DMSO) and the hemp seed oil 0.5% (v/v) both at 24h and 48h (Fig 2).

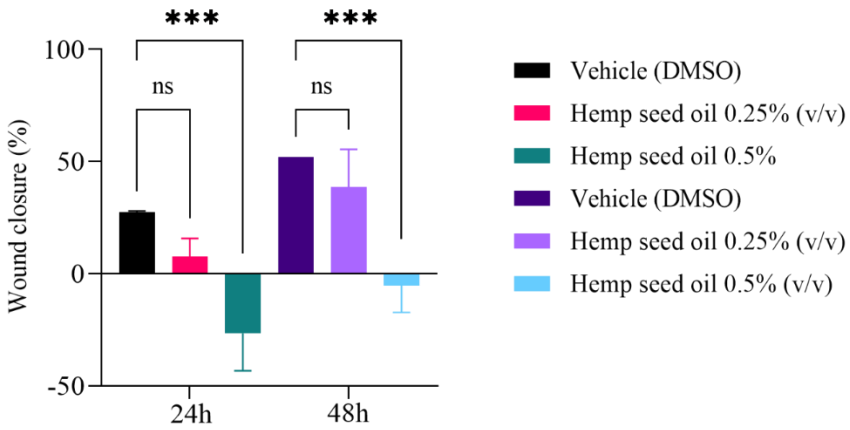

Fig. 2 The statistical analysis compared the treatments (0.25% oil and 0.5% hemp seed oil) to the vehicle (DMSO), with a significance level of $p < 0.0005$.

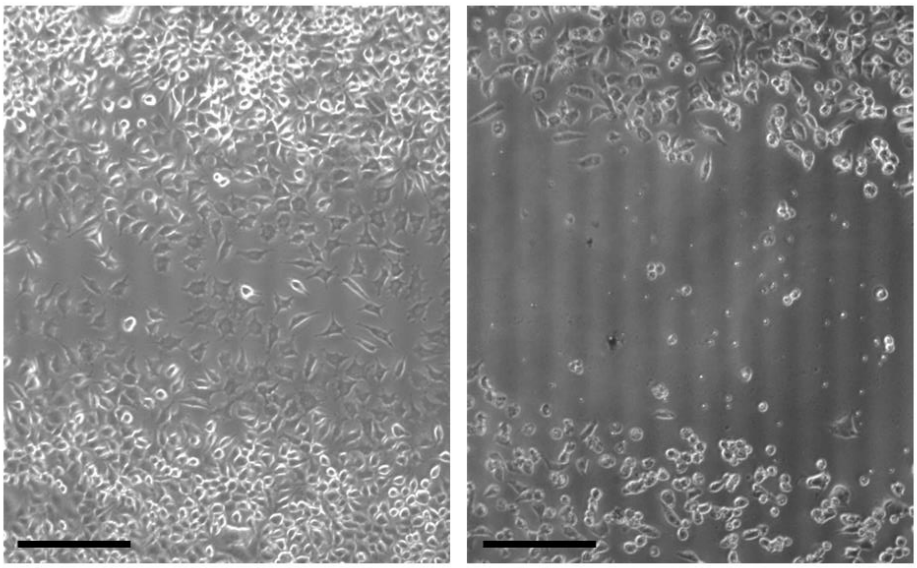

Fig 3: Representative images comparing L929 cells treated with FBS 2% (left) and hemp seed oil 0.5% (right) after 48 hours (scale bar 200 µm).

At the 0.5% hemp seed oil concentration, values were observed to be lower than the 0h threshold of both the vehicle and DMEM controls. As shown in the representative images in figure 3, after 48 h of exposure, a clear morphological change was evident in the L929 cells treated with hemp seed oil 0.5%. In comparison to the cells treated with FBS 2%, the hemp seed oil-treated cells lost their typical fibroblastic shape and stopped proliferating.

4. Discussion

Dermal wound healing is a complex process that involves key events such as angiogenesis and extracellular matrix remodeling [24]. The mitogenic response plays a vital role in wound repair, with fibroblasts being central to this process. They contribute to wound contraction, fibroplasia, extracellular matrix production, and the reduction of inflammation, all of which support tissue regeneration [25]. Cannabis extracts, rich in cannabinoids, carotenoids, chlorophyll, flavonoids, terpenes, and phytosterols, exhibits potent wound-healing properties. Hemp's extracts combination of antimicrobial, anti-inflammatory, and antioxidant properties makes it highly effective in wound care [26]. In our previous study, promising results were observed regarding the antimicrobial activity of hemp seed oil extract at the same concentrations tested in this research. Therefore, we decided to assess its potential wound-healing properties in the present study. However, this preliminary in vitro investigation did not show any significant wound-healing effects of hemp seed oil. The results suggest that treatment with hemp seed oil at various concentrations does not notably enhance wound healing. The wound-healing activity of cannabis and its extracts is generally attributed to cannabinoids such as CBD and THC [27]. Specifically, previous studies have linked the wound-

healing properties of hemp seed oil to its CBD content [28]. However, the hemp seed oil extract used in this study is devoid of both CBD and THC, which may explain the absence of detectable wound-healing activity. Additionally, the 0.5% concentration of hemp seed oil tested in this study showed an inhibitory effect on wound healing. As seen in the images, cells treated with hemp seed oil at the highest concentration displayed significant morphological changes and were in a semi-adherent state, likely due to the incorporation of lipid particles from the oil by the fibroblasts. To the best of our knowledge, this is the first study to evaluate the wound-healing potential of hemp seed oil on the L929 fibroblast cell line. The selection of the L929 murine fibroblast cell line is based on its extensive characterization in scientific literature and its frequent use in studies investigating the wound-healing potential of plant-derived compounds or extracts [1], [7]. Moreover, our previous study demonstrated that the concentrations of hemp seed oil used in this work do not exert cytotoxic effects on the L929 fibroblast cell line. As this is a preliminary study, further research is needed to fully evaluate the wound-healing potential of hemp seed oil. Future studies should explore a broader range of cell types and test varying concentrations of hemp seed oil to identify the most effective dosages. Additionally, investigating the mechanisms of action and how hemp seed oil interacts with other biological factors involved in wound healing will provide deeper insights into its therapeutic effects. These expanded investigations will help clarify the role of hemp seed oil in tissue repair and its potential for clinical applications.

The reported results constitute a preliminary research draft

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Chapter 7: Conclusions & future perspectives

Antibiotic resistance is a significant global health threat, exacerbated by the rising prevalence of multidrug-resistant (MDR) bacterial pathogens in both animals and humans, coupled with the limited efficacy of conventional antimicrobial treatments. This highlights the urgent need for novel therapeutic approaches for infectious diseases. Of particular concern is the spread of MDR bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-resistant *Staphylococcus pseudintermedius* (MRSP) and *Pseudomonas aeurginosa*. The EFSA Panel on Animal Health and Welfare has identified *S. pseudintermedius* and *P. aeruginosa* as priority antimicrobial-resistant pathogens in companion animals across the EU.

Given the high incidence of skin diseases in small animals that often require antibiotic treatment, alternative treatments are critically needed. In this context, plant-derived products, with their well-documented antimicrobial properties, have garnered attention. Among these, hemp (*Cannabis sativa L.*) and its derivates have shown promising pharmacological properties, including antibacterial activity against Gram-positive bacteria such as MRSA and MRSP.

The antimicrobial efficacy of hemp is largely attributed to cannabinoids, especially cannabidiol (CBD) and tetrahydrocannabinol (THC), which exhibit well-documented antibacterial effects.

Hemp extracts are rich in bioactive compounds that act synergistically, making it harder for bacteria to develop resistance, one of the primary challenges associated with conventional antibiotics.

Hemp seed oil, extracted from the seeds of *Cannabis sativa* L., is valued for its nutritional and health-promoting properties. However, its specific antibacterial activity remains poorly understood, with limited studies

focusing on its effects against Gram-positive and Gram-negative bacteria, often using qualitative methods.

Our study investigates the antibacterial activity of hemp seed oil against *S. aureus*, *S. pseudintermedius*, and *P. aeruginosa*, isolated from dogs. Additionally, we explored its potential synergistic effects with antimicrobial agents commonly used in topical veterinary treatments. Our results demonstrated that hemp seed oil extract exhibits antimicrobial activity against the tested bacterial strains. Importantly, the concentrations of the extract showing antimicrobial effects did not display cytotoxicity toward murine fibroblast cell lines included in the study.

Cannabis extracts are also studied for their wound-healing potential, thanks to their rich composition of bioactive compounds. Previous studies have linked the wound-healing properties of hemp seed oil to its CBD content. While earlier research confirmed the antimicrobial efficacy of hemp seed oil extract, this study did not observe significant wound-healing effects in vitro. This result is likely due to the absence of CBD and THC, key cannabinoids known to facilitate wound healing, in the tested extract. The findings presented in this thesis underscore the need for further in-depth research to fully explore the antimicrobial and wound-healing potential of hemp extracts.

Such investigations should aim to identify not only the primary active components but also their mechanisms of action. Additionally, further studies are necessary to explore the wound-healing potential of hemp extracts. Future studies should aim to identify the primary active components and elucidate their mechanisms of action. Expanded research should also investigate a broader range of cell models and test varying concentrations to determine optimal dosages. Additionally, understanding the molecular pathways and biological interactions influenced by hemp

extracts will provide valuable insights. These efforts are crucial to clarify the therapeutic potential of hemp extracts in tissue repair and to evaluate their suitability for clinical applications, particularly in veterinary medicine.

ADDENDUM

List of publications

1. Siddiqui SA, **Toppi V**, Syiffah L. "A comparative review on Ayam Cemani chicken – A comparison with the most common chicken species in terms of nutritional values, LCA, price and consumer acceptance" *Tropical Animal Health and Production*, 2024;
2. Musa L*, **Toppi V***, Stefanetti V, Spata N, Rapi MC, Grilli G, Addis MF, Di Giacinto G, Franciosini MP. "High biofilm-forming Multidrug-Resistant Salmonella Infantis strains from the poultry production chain" *Antibiotics*, 2024;
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3. Pearce R, Pirolo M, Goecke NB, **Toppi V**, Good L, Guitian J, Guardabassi L. "Imported seafood is a reservoir of CTX-M-encoding genes of high clinical relevance" Submitted to *International Journal of Food Microbiology* 2024;
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5. **Toppi V**, Scattini G, Musa L, Stefanetti V, Pascucci L, Chiaradia E, Tognoloni A, Giovagnoli S, Franciosini MP, Branciari R, Casagrande Proietti P. "Evaluation of β-lactamase enzyme activity in outer membrane vesicles (OMVs) isolated from extended spectrum β-lactamase (ESBL) *Salmonella Infantis* strains" *Antibiotics*, 2023;
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Participation at national and international congresses

Oral presentations

1. **Toppi V.**, Pirolo M., Rampacci E., de Benedetti M., della Rocca G., Di Salvo A., Guardabassi L., Casagrande Proietti P. "Antimicrobial activity of hemp extract seed oil against bacterial pathogens in dogs", 77° Convegno SISVET, 12/06/2024, Parma (IT);
2. **Toppi V.**, Scattini G., Musa L., Stefanetti V., Pascucci L., Chiaradia E., Tognoloni A., Giovagnoli S., Franciosini MP., Casagrande Proietti P. "Methods for OMVs isolation and evaluation of β-lactamase enzyme activity in Salmonella Infantis", 22-24/04/2024, Torino (IT);
3. Musa L., Stefanetti V., **Toppi V**., Spata N., Di Giacinto G., Franciosini MP., Grilli G., Casagrande Proietti P. "High capacity of biofilm formation in multidrug-resistant *Salmonella* Infantis strains and morphotype association from poultry production chain" 5th International conference of the European college of Veterinary microbiology, 21-23/09/2023, Bled (Slovenia);
4. **Toppi V**., Rampacci E., Musa L., della Rocca G., Di Salvo A., De Benedetti M., Di Giacinto G., Franciosini MP., Casagrande Proietti P. "Antimicrobial activity of Hemp extract seed oil against *Staphylococcus pseudintermedius* and *Pseudomonas aeruginosa* strains isolated from pyoderma and external otitis in dogs", 76° Convegno SISVET, 21-23/06/2023, Bari (IT);

5. **Toppi V**., Scattini G., Musa L., Stefanetti V., Pascucci L., Chiaradia E., Tognoloni A., Giovagnoli S., Franciosini MP., Casagrande Proietti P. "Evaluation of β-lactamase activity in Outer Membrane Vesicles (OMVs) isolated from Extended Spectrum β-lactamase (ESBL) *Salmonella* Infantis strains", International conference on one health antimicrobial resistance, 18-20/04/2024, Copenhagen (DK);
6. Musa L., Casagrande Proietti P., Stefanetti V., **Toppi V**., Gobbi M., Costantini I., Aisa F., Brescia M., Franciosini MP. "Indagini preliminari" sul ruolo rivestito da volatili selvatici come potenziali reservoir di microorganismi antibiotico-resistenti", 28/10/2022, VII simposio scientifico della società italiana di patologia aviare (SIPA), Forlì (IT);
7. Musa L., Casagrande Proietti P., Marenzoni ML., Shtylla Kika T., Stefanetti V., Ranucci D., **Toppi V**., Franciosini MP. "Minimum of inhibitory concentrations (MIC) of selected antimicrobials against commensal *E. coli* from conventional, organic, and antibiotic-free broiler chickens in farms and in slaughterhouses". 3rd International Conference of the European College of Veterinary Microbiology. 16-17 ottobre 2021, Webinar;
8. Musa L., Casagrande Proietti P., Stefanetti V., **Toppi V**., Marenzoni MP., Shtylla Kika T., Blasi F., Magistrali CF., Branciari R., Franciosini MP. "Phenotypic and genotypic study on susceptibility to colistin in *E. coli* and *Salmonella* Infantis isolated in meat chickens" VI Simposio Orale SIPA, 19 Novembre 2021, Bologna (IT);
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11. **Toppi V.,** Rampacci E., della Rocca G., Di Salvo A., De Benedetti M., Casagrande Proietti P. "Study of Antimicrobial activity of Hemp extract seed oil against *Staphylococcus*

pseudintermedius strains and *Pseudomonas aeruginosa* strains isolated from pyoderma and external otitis in dogs", 15-16/02/2022, Londra (UK);

Poster presentations

1. Marini D., Di Giacinto G., **Toppi V**., Di Nicola MR., Musa L., Massacci FR., Binucci G., Casagrande Proietti P., Marenzoni ML., Di Tizio L., Di Toro F., Carafa M., "Exploring suitable photo identification region to monitor pathogens and environmental antimicrobial resistance in wild snakes annually captured in the San Domenico Abate workship of Pretoro", XV Congresso nazionale della societas herpetologica italica, 21/09/2024, Perugia (PG);
2. Di Giacinto G., Musa L., Ranucci D., Cai D., **Toppi V.,** Spata N., Casagrande Proietti P. "Studio preliminare della suscettibilità antimicrobica di *E. coli* in 4 aree della provincia di Perugia" III congresso nazionale filiere delle carni di selvaggina selvatica, 10-12/05/2023, Foligno (IT);
3. **Toppi V**., Rampacci E., Musa L., della Rocca G., Di Salvo A., De Benedetti M., Franciosini MP., Casagrande Proietti P. "Hemp extract seed oil antimicrobial activity against *Staphylococcus pseudintermedius* and *Pseudomonas aeruginosa* strains isolated from pyoderma and external otitis in dogs" International conference on one health antimicrobial resistance, 18-20/04/2023, Copenhagen (DK);
4. **Toppi V**., Scattini G., Musa L., Pascucci L., Chiaradia E., Tognoloni A., Franciosini MP., Casagrande Proietti P. "*Salmonella* Infantis releases Outer Membrane Vesicles (OMVs) including β-lactamase enzymes during their biogenesis", 50° Congresso nazionale della società italiana di microbiologia, 18-21/09/2022, Napoli (IT);
5. **Toppi V**., Musa L., Scattini G., Pascucci L., Franciosini MP., Casagrande Proietti P. "A preliminary study on *Salmonella* Infantis: outer membrane vesicles (OMVs) isolation and evaluation of β-lactamase activity", 3rd international conference of the european college of veterinary microbiology, 16-17/10/2021, webinar.