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One Health in Animal Science - Circulation of antimicrobial resistance between the domains of health

MASTER'S THESIS (MSC)

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1. INTRODUCTION

Antimicrobials have transformed healthcare practices globally since Alexander Fleming discovered penicillin in 1928. Many antibiotics are used to treat infectious diseases in human and animal medicine (Diarra and Malouin, 2014). The agents used in treating animals and humans often belong to the same classes of antibiotics with similar modes of action and bacterial cell targets. Bacteria acquiring resistance to these drugs in animals might be transferred to humans or spread their mechanisms of resistance, which may eventually be found in human pathogens. The bacteria developing resistance can cause equal loss of therapeutic efficacy in veterinary as well as in human medicine, establishing a worldwide health challenge. Continuous advancement in public health, and decreased mortality and morbidity rates related to infectious diseases has been accredited to the discovery of antibiotics.

These days, antimicrobial resistance (AMR) has become a global health challenge to humanity because the fatal cases of antimicrobial resistant pathogens increase continuously. AMR impacts everyday life all over the world. The resistant bacterial strains frequently cause epidemic outbreaks, which burden the healthcare systems and, in many cases, the economies (WHO, 2016). Patients infected with drug-resistant bacteria require more health and financial support for treatment. Excess healthcare system costs ranged from non-significant to $1 billion per year, whereas economic direction varied to over $3 trillion in GDP loss (O'Neil, 2016). The number of deaths associated with resistant bacterial strains was estimated by a statistical model to be roughly 5,000,000 in 2019 (Antimicrobial Resistance Collaborators, 2022).

Recently, due to urban population expansion, fragmentation, encroachment and loss of natural habitats, there is an increased interaction between humans, domestic animals, and wildlife. For this reason, One Health approach is the best approach to examine antimicrobial resistance phenomenon (White & Hughes, 2019). This point of view recognizes that different domains of health are integrated. Therefore, human, livestock, wildlife, and environmental health should be key precedence in reducing antimicrobial resistance.

The S*taphylococcus* genus including *S. aureus* are ubiquitous. They can be found everywhere in the environment including the human food chain. They can also be found in natural microbiota without causing illness and they can similarly be detected in clinically healthy animals and humans. However, they can also be opportunistic pathogens, leading to mild and severe infections. It has been demonstrated in numerous studies and articles that *S. aureus* and several coagulase-negative *Staphylococcus* species (CoNS) have a remarkable ability to develop resistance to any antibiotic it is exposed to. Consequently, the continuous spread of AMR-resistant staphylococci presents a significant One Health challenge (Algammal et al., 2020; Khamesipour et al., 2022). Improper use of antimicrobials in human communities or animal populations can lead to the emergence of AMR, which can circulate between the three interdependent health domains. The One Health approach recognizes that healthy humans and animals depend on a healthy ecosystem. This healthy ecosystem allows for treating common pathogens from all three domains using available drugs (Hernando-Amado et al., 2019; Koch et al., 2017).

2. REVIEW OF LITERATURE

In addition to their therapeutic use, research by Diarra and Malouin (2014) showed an unexpected application of antibiotics in the growth enhancement of food animals. A positive effect of antibiotics on growth was observed in the 1940s when animals fed dried mycelia of *Streptomyces aureofaciens* containing chlortetracycline residues showed improved performance (Castanon, 2007). Another research illustrated that antibiotics often improve poultry growth performance by increasing feed conversion efficiency and reducing disease incidences (Castanon, 2007; Gadde et al., 2021). Due to antimicrobial resistance and antibiotic residues in the food chain and the environment, there has been an increase in global health apprehension regarding the use of antibiotics in poultry diets for growth promotion and prophylaxis. (Muaz et al., 2018; Wales et al., 2019). This practice has been linked to the emergence and spread of antibiotic- resistance and genes posing a significant threat to modern medicine (Dutil et al., 2010). This concern has been a topic of debate, leading to banning antimicrobial use as growth promoters in many countries, with the European Union taking the first initiative in the 2006 debate (Vaughn & Copeland, 2004).

While some bacteria naturally develop resistance over time, largely as an inherent process (D'Costa et al.,.2011), acquired resistance is primarily a result of antibiotics being underused, overused, or misused (Davies, 1996; Lammie & Hughes, 2016). Antibiotic consumption in all healthcare settings is undeniably high, which is a major contributing factor to antimicrobial resistance (Laxminarayan et al., 2020). For example, transmission events of *Staphylococcus aureus* between humans and livestock have been documented, and the widespread use of antibiotics in both humans and veterinary medicine may create a selective pressure that promotes antibiotic resistance (Haag et al., 2019; Richardson et al., 2018).

With methicillin-resistant S*taphylococcus aureus* (MRSA), it becomes very difficult to treat infections. The resistance predominantly develops from acquiring *mec A* gene, which codes a modified penicillin-binding protein (PBP2a) with diminished affinity for beta-lactam antibiotics. Livestock became recognized as a significant reservoir of (MRSA) in 2003. Livestock-associated MRSA, particularly clonal complex 398 (CC398), comprise 25% of all surveillance isolates.

The wild boar (*Sus scrofa*) is extremely adaptable and widely distributed traversing urban environments around the globe. Recently, wild boars have increased in numbers and range in

Europe, and this makes them one of the most abundant ungulate species (Massei et al., 2015; Pittiglio et al., 2018). In the perspective of AMR, wild boar represents an ideal subject for understanding AMR's emergence, spread and persistence at the interface between wildlife and livestock.

Their widespread distribution, extensive home ranges, limited exposure to antibiotics in natural settings, and overlapping habitats with livestock and humans position them as crucial links between human-influenced areas and natural ecosystems (Macdonald & Laurenson, 2006). Additionally, wild boar hunting and meat processing in various regions worldwide serve as a direct avenue for transmitting resistance bacteria and associated genes between wild boar and humans. With the increased number of wild boars in several European countries, the interaction between wild boars, livestock, and humans is anticipated to escalate (Castillo-Contreras et al., 2018). These observations underscore the potential of wild boars as significant reservoirs and disseminators of bacteria carrying antimicrobial resistance, thereby presenting additional challenges to public health.

2.1 Methicillin- resistant *Staphylococcus aureus*

S. aureus is Gram-positive bacteria that is catalase-positive and frequently lives on the skin and mucosa of man and non-human animals (Stratton et al., 1982; Huebner et al., 1999; Wertheim et al., 2005). The anterior nares of humans are the most preferred colonization sites, with approximately up to 30% of human population being asymptomatically and permanently colonized (Tong et al., 2015; Wertheim, 2005). Despite being deemed a commensal organism, *S.aureus* is also a facultative pathogen capable of causing various diseases, from mild skin issues to acute and potentially life-threatening infections (Lowy, 2003). In its commensal state, *S.aureus* establishes residence in its host without causing harm. Depending on the duration of carriage, hosts can be classified as persistent carriers, intermittent carriers, or non-carriers altogether, with transient colonization lasting no more than approximately two weeks (van den Broek et al., 2009). However, nasal swabs from persistent carriers often yield consistent MRSA strains over time. Despite the organism's facultative pathogenic nature, persistent carriers face an increased risk of clinical manifestation of their infection (van den Broek et al., 2009).

Penicillin-resistant *S. aureus* developed just a few years following the introduction of penicillin in the 1940s (Barber 1949). Plasmids with the β-lactamase gene (blaZ), which produces an enzyme that breaks down the β-lactam ring in penicillin and other antibiotics, were taken up, resulting in resistance (Lowy, 2003; Novick, 1963). The resistance was conferred through the uptake of plasmids containing the β-lactamase gene (*blaZ*), which produces an enzyme capable of degrading the β-lactam ring in penicillin and other antibiotics (Lowy, 2003; Novick, 1963). The β-lactam ring of penicillin works by binding to penicillin-binding proteins (PBPs), needed for cross-linking the peptidoglycan chain in the bacterial cell wall (Ghuysen 1994).

Two decades later, in 1960, methicillin was introduced as an antibiotic designed to resist β-lactamase. A more dangerous form of staphylococcus known as methicillin-resistant *S.aureus* (MRSA) strains emerged (Barber 1961; Jevons 1961). This new resistance mechanism stemmed from acquiring the *mecA* gene, which codes for penicillin-binding protein 2a (PBP2a). PBP2a exhibits an altered structure, rendering it less susceptible to β-lactam antibiotics (Hartman et al., 1984). It is widely recognized that widespread use of antibiotics for preventive and therapeutic functions generates selective pressure that drives the emergence of resistant strains. This resistance is primarily observed in domestic animals. In pigs and poultry antibiotics are commonly used as growth promoters, which further worsens the issue. This resistance occurrence is not limited to domestic animals but also to other wild animals inhabiting the same environments and sharing resources (Gortázar et al., 2016).

Subsequently, during the 1980s and the 1990s, MRSA strains spread worldwide, demonstrating resistance to various drugs, and becoming key contributors to nosocomial infections (Lowy 1998). Because of their prevalence in health care settings and hospitals, these MRSA variants were coined hospital-acquired MRSA (HA-MRSA). With the emergence of HA-MRSA the hospitals put into operation control measures to reduce nosocomial spread of MRSA (Saravolatz et al., 1982). In numerous countries, these interventions seemed to be effective in reducing the prevalence of HA-MRSA. Nonetheless, there was a surged in infection caused by novel MRSA strains in communities outside the hospital settings during the 1990s (CDC 1999; Herold et al., 1998). These strains were capable of fast dissemination between healthy people, and they were termed as community acquired MRSA (CA-MRSA) (Chambers & DeLeo, 2009).

In the Netherlands, a novel MRSA strain was discovered colonizing a pig farmer's daughter in the year 2004 (Voss et al., 2005). Both parents and a pig on the family farm carried the identical strain, characterized by the presence of the *mecA* gene. The strain was different from the conventional HA-MRSA and CA-MRSA strains, because it could not be classified under the standard pulsed-field gel electrophoresis (PFGE) method with restriction endonuclease *SmaI*. This was the first research that demonstrated direct MRSA transmission between animals and humans, although prior studies had hinted at a connection between animal and human colonization. Subsequent investigations in other regions collaborated on these findings. To have a distinction among the subset of MRSA strain acting as reservoirs in livestock animals the term livestock-associated MRSA (LA-MRSA) was invented (Reischl et al., 2009).

LA-MRSA are notorious strains with the potential to spread between different animal species and to humans with close contact with colonized animals, for example the veterinarians and farm workers. Additionally, colonized humans can serve as a transmission source, spreading LA-MRSA to other humans and between different animal environments. Similarly, humans also serve as a reservoir for transmitting *S. aureus* to vertebrate animals. Amphixenoses are infections that can be present in both humans and animals and transmitted in both directions, for instance *Staphylococcus aureus* infection (Rossi et al., 2017). In Belgium in the early 1970s, the initial case of MRSA colonization was reported, affection the milk of cows with bovine mastitis (Devriese et al., 1972). Despite most isolates being like common bovine strains, the likelihood of a new MRSA strain acquired by the farmer being transmitted to animals seems more plausible due to observed antimicrobial characteristics. Since then, MRSA colonization has been documented in dogs, cats, horses, cattle, pigs, rabbits, and poultry (Moreno-Grúa et al., 2018; Weese, 2010; Werckenthin et al., 2001). Instances have even been reported in wild birds such as magpies and vultures (Mama et al., 2019). The first documented case of animal-to-human transmission of *S. aureus* was reported in dairy sheep (Vautor et al., 2003). Shortly after, the first instance of MRSA transmission from animals to humans and subsequent human-to-human transmission of the same strain was reported. Interactions between humans and their pets can result in cross-species transmission, where household animals may carry the same strains of *S. aureus* as their human counterparts (Hanselman et al., 2009). Despite the potential for *S. aureus* colonization in domestic and livestock animals, intensive farming systems, such as housing pigs, veal calves, and broilers, are identified as the primary reservoirs of LA-MRSA in affected regions (EFSA 2009).

In general, transmission of *S. aureus* happens through direct contact with colonized skin or mechanical vectors (Boyce et al. 1997). The spread of MRSA can happen easily within the livestock systems. They can also enter the farm through several route, like movement of colonized animals between farms, direct contact with individuals carrying the bacteria, and contact with contaminate transport vehicles (van Duijkeren et al., 2008; Broens et al., 2012; Grøntvedt et al., 2016).

Indirect transmission pathways also exist, with environmental factors playing a significant role. Dust, for instance, can serve as a carrier of *S. aureus* for extended periods, facilitating its spread between farms (Feld et al., 2018). Dust particles laden with staphylococci can be transported on vehicles, and farmworkers may accidentally carry MRSA-contaminated dust home on their clothing. Moreover, wind dispersal can transport dust particles over considerable distances, potentially spreading staphylococci to remote locations; airborne MRSA transmission has been observed in hospital settings and among farm personnel (Bos et al., 2016; Seedorf et al., 2005).

In addition to direct contact and environmental exposure, MRSA transmission can occur through food production systems. Animals may act as reservoirs for antimicrobial-resistant bacteria, and products such as meat, milk, and eggs could serve as vehicles for transmitting these bacteria to consumers (Pondit et al., 2018; Ge et al., 2017). Nonetheless, the risk of acquiring MRSA through food consumption appears relatively low (EFSA 2009). Pig farmers and veterinarians attending farms face an elevated risk of carrying LA-MRSA (Lewis et al., 2008), with surveys indicating prevalence rates as high as 77%–86% among individuals working in MRSA-positive farm environments (Cuny et al., 2009; van den Broek et al., 2009).

While colonization appears to be transient in many cases (Sieber et al., 2019), data also suggest instances of long-term persistence (Köck et al., 2012). Consequently, it's not unexpected that LA-MRSA CC398 has infiltrated the human healthcare system in certain European countries, initially identified as an occupational hazard for individuals involved in animal husbandry (Lewis et al., 2008). However, recent findings indicate the spread of LA-MRSA among individuals lacking direct contact with livestock, likely stemming from the bacteria's recent adaptation to the human host (Sieber et al., 2019). Beyond its zoonotic

potential, MRSA infections present treatment challenges due to frequent resistance to multiple classes of antibiotics.

The involvement of other host species remains unclear. A comprehensive study of *S. aureus* strains from European wildlife, including cervids, has recently been conducted, revealing significant genetic diversity and relatively low levels of antimicrobial resistance (Monecke et al., 2016). Recent reviews have focused on MRSA in wildlife without prior antibiotic exposure (Heaton et al., 2020; Silva et al., 2020), indicating that most MRSA strains isolated from wild animals are *mecC*-positive. The spread of this resistance mechanism (García et al., 2020; Shore et al., 2011) among free-living animals and in river waters (Porrero et al., 2014) suggests that the natural environment might serve as a reservoir for the *mecC* gene. Certainly, the co-colonisation of hedgehogs with *S. aureus* and penicillin-producing dermatophytes exerts selective pressure favouring *mecC* in the wild (Larsen et al., 2022; Dube et al., 2021; Smith & Marples, 1965).

2.2 *Staphylococcus* species in deer

Red deer (*Cervus elaphus*) populations are increasing in density and range across Europe (Lovari et al., 2018), aided by their ecological adaptability to a variety of habitats, including forests, grasslands, and agricultural landscapes. Given that the wild animals are not exposed to antibiotics, the presence of livestock-associated *S. aureus*/MRSA influx on wildlife has gained attention. This in turn leads to the recognition that there is need of baseline data on untreated free-living animals and the importance of monitoring the potential impact of human on these strains (Heaton et al., 2020; Monecke et al., 2016). The interactions between natural and human-influenced environments are crucial in disseminating antimicrobial resistance within wild ecosystems. Despite existing knowledge, key questions remain unanswered regarding the impact of land-use-related factors on the spread of antimicrobial-resistant staphylococci in European wild game animal populations.

In a study conducted to assess the occurrence of Staphylococcaceae with reduced susceptibility to cefoxitin in hunted fallow deer, red deer, roe deer, and wild boar in Brandenburg, Germany, the focus was on utilizing open-source data to analyze the extent and intensity of land use, particularly related to settlements or animal husbandry. Over three years,

371 nasal swabs were collected, with 45 (12.1%) testing positive for Staphylococcaceae exhibiting a non-wild-type phenotype for cefoxitin (>4 mg/Liter). The detection rates varied among species, with fallow deer at 21.8%, red deer at 22.7%, roe deer at 11.5%, and wild boar at 5.0%. Statistical significance was observed in the prevalence differences between fallow deer and wild boar, as well as between red deer and wild boar. However, differences in detection rates were not associated with hunting season, sex, or age (Mateus-Vargas et al., 2022).

The study's results indicated that the detection rates of Staphylococcaceae with reduced susceptibility to cefoxitin varied among animal species in the studied hunting districts. Statistical analyses and land use data revealed that a higher density of cattle or poultry in a county may correlate with an increased detection rate in roe deer or wild boar, respectively. Additionally, positive correlations were found between the prevalence of non-wild-type Staphylococcaceae in roe deer or fallow deer and the proportional extent of surface water bodies in the corresponding area (Mateus-Vargas et al., 2022). This approach provides a foundation for a risk-oriented evaluation of the impacts of human activities on the epidemiology of transmissible microorganisms at the human-animal-environment interface, including antimicrobial-resistant bacteria.

In recent studies, *S. aureus*, a pathogen with the potential to affect various host species, has been observed to transmit between humans and animals, as well as among different animal species. Luzzago et al. (2022) conducted a study involving 284 free-living red deer in the Central Italian Alps to investigate the prevalence and molecular characteristics of *S. aureus* in nasal and intestinal samples, considering host characteristics and environmental factors. The study revealed a prevalence of 90% in nasal swabs, 26.2% in rectal swabs, and 10.7% in faecal samples. Calves were more likely to be intestinal carriers of *S. aureus* than adults, particularly among females in faecal samples. The most prevalent lineage detected was Clonal Complex (CC) 425, commonly found in domestic and free-living animals, followed by CC2671 and CC350. CC425 and CC350 showed a high incidence of the phage-borne virulence factor *lukM/lukF*-P83. Other lineages, sporadically detected in red deer faeces, included CC7, CC9, CC121, and CC707, which are known to occur in both humans and animals and carry genes associated with penicillinase operon and macrolide resistance (CC9 and CC121). However, methicillin-resistance genes *mecA* and *mecC* were not identified. These findings suggest that free-living red deer may serve as a reservoir for *S. aureus* in Alpine habitats.

A group study aimed to assess the presence of methicillin-resistant *Staphylococcus* (MRS) strains in non-managed wild ungulates found in a typical Mediterranean forest in Spain. Nasal swab samples were collected from 139 animals, including 90 wild boars (*Sus scrofa*), 42 red deer, and 7 fallow deer (*Dama dama*). The samples were later pre-enriched in BHI+ NaCl (6.5%) for 24 hours at 37°C, followed by seeding in Columbia blood agar for another 24 hours at 37°C. PCR analysis was performed to detect the presence of the *mecA* gene, initially from the confluent colonies and then from individual colonies (Rey Pérez et al., 2021). From the colonies that were obtained, 10 tested positive for *mecA* and just 7 showed phenotypic resistance to oxacillin/cefoxitin, suggesting methicillin resistance. All identified MRS strains belonged to the *Staphylococcus sciuri* group, with no MRSA detection. Moreover, a significant proportion of the MRS strains displayed resistance to other antimicrobials, particularly β-lactams, gentamicin, fusidic acid, and quinupristin-dalfopristin. However, this resistance did not consistently correlate with their genetic makeup (Rey Pérez et al., 2021). Genetic profiling revealed the clustering of the seven strains based on bacterial species rather than the animal source or geographical location. Notably, *SCCmec* type III, commonly found in animals and humans, was detected in three strains obtained. This study also highlights the role of the investigated wild ungulates as potential reservoirs of multi-resistant MRS strains. Given their characteristics, these strains have the potential to spread easily to other wild or domestic animal species and, ultimately, to humans through various means, including animal products (Rey Pérez et al., 2021).

2.3 *Staphylococcus* species in wild boar

The distribution and population of wild boars across Europe have significantly increased in recent decades. With wild boars exhibiting scavenging feeding behaviors, as well as considering the dynamics of AMR, this species is ideal for investing AMR's emergence, spread, and endurance at the interface of livestock, humans, and wildlife interactions. In a study done in Spain, 371 wild boar nasal samples were collected to determine the prevalence and diversity of coagulase-positive staphylococci (CoPS) in nasal samples from healthy wild boars, to study their resistance phenotypes/genotypes and to control for the prevalence of MRSA-ST398 (Mama et al, 2019). The prevalence of CoPS carriage (including

Staphylococcus aureus, *Staphylococcus hyicus*, and *Staphylococcus pseudintermedius*) in wild boar was found to be 17.8%, with *S. aureus* being the most prevalent at 13.7%, followed by *S. hyicus* at 2.7%, and *S. pseudintermedius* at 1.6%. Among *S. aureus* isolates, 74.5% showed susceptibility to all tested antimicrobials, except for one strain identified as MRSA (lineage ST398-t011-*agrI*), which carried *blaZ*, *mecA*, *tet*(M), and *tet*(K) genes. A total of 22 *spa*-types and 17 sequence types (STs) were identified among the *S. aureus* isolates, including ST398/CC398 (n = 1), ST2328-ST133/CC133 (n = 20), ST425/CC425 (n = 7), ST5/CC5 (n = 5), ST1/CC1 (n = 3), ST130/CC130 (n = 2), and ST88/CC88 (n = 1). Among the six *S. pseudintermedius* isolates recovered, two *spa*-types (t02, t15) and four STs (ST455, ST796, ST797, ST798) were detected, all of which carried the *lukF/S-I* and *siet* virulence genes. All *S. hyicus* isolates tested were susceptible to the antimicrobials used in the study (Mama et al., 2019).

In another study conducted to investigate *Staphylococcus aureu*s carrying gene in animals and urban wastewaters in Spain, *mecC* was identified in total of four isolates, with one isolate from a wild boar, two from fallow deer, and one from urban wastewater, accounting for 1% of the 361 tested isolates. Among the three isolates recovered from animals, they exhibited susceptibility to all antimicrobial drugs except for β-lactams and oxacillin, with minimum inhibitory concentrations (MICs) ranging from 0.5 to 1 mg/L for oxacillin but resistance to penicillin with MICs ranging from 0.5 to 2 mg/L. Two isolates demonstrated resistance to cefoxitin, with MICs of 8 and 16 mg/L, while the third isolate was susceptible with an MIC of 4 mg/L. The wastewater isolate showed resistance to penicillin (MIC 2 mg/L) and erythromycin (MIC 16 mg/L) but susceptibility to all other antimicrobial drugs tested, including cefoxitin (MIC 4 mg/L) and oxacillin (MIC ≤0.25 mg/L), (Bonnedahl et al., 2014).

2.4 Aim of the study

This study aimed to collect data on the occurrence and antibiogram of the member *Spathylococcus* genus in a natural habitat shared with different game species. The hypothesis was that different species could be carriers of the same bacteria, and similar resistance patterns could be observed. The presence of resistant staphylococci in nature can support the assumption that the AMR spread has no limit between humans and wild animals. The study

aims to demonstrate the benefits of a One Health approach in estimating the damage to ecosystem health attributable to human activities.

3. Experimental study

3.1 Material and methods

3.1.1 Sampling site

The study was carried out with a total of 19 red deer (*Cervus elaphus*) (RD) and 19 wild boar (*Sus scrofa*) (WB) samples. The samples were collected in the 2023-2024 hunting season from a hunting area in Hungary. The hunting site (Zselic Forestry) was located in the southwestern part of Hungary and managed by SEFAG PLC (**Figure 1**). The most important game species are red deer, wild boar, and of lesser importance fallow deer and roe deer. The animals were harvested on driving hunt events; the purpose of the shoot was not to conduct the investigation but to fulfil the authorized wildlife management plan.

The forestry can be characterized by a 145–276 m high average altitude above sea level. The climate of this area is typically sub-Mediterranean (warm summer, relatively mild winter) with some submontane habitat patches (10°C as the mean annual temperature and 630–800 mm annual precipitation). Forest cover is more than 70% with a 6000-ha core monoblock forest. The main tree species are oaks (*Quercus* spp.), limes (*Tilia* spp.), beech (*Fagus sylvatica*), hornbeam (*Carpinus betulus*) and Robinia (*Robinia pseudo acacia*) to a smaller extent. On the peripheries, agricultural settlements, some small villages, a sheep farm (approximately 800-850 sheep), and a red deer farm (approximately 1100 deer) can be found. The most populous town of Somogy County, Kaposvár (population: 59300), is located about 15 km north of the study site. More and more people visit the area for recreational purposes.

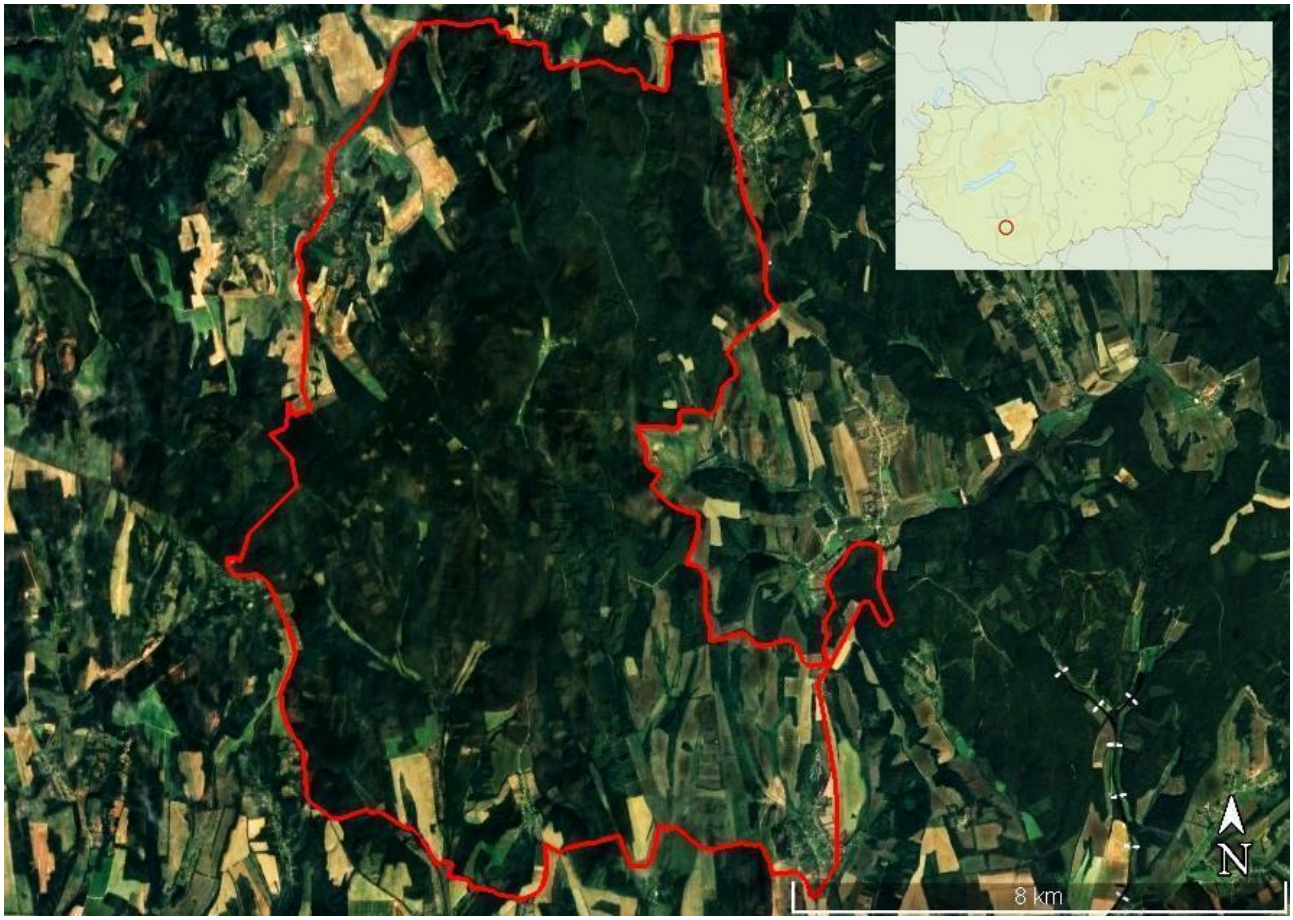

Figure 1. Localization of sampling site in Hungary

3.1.2 Sampling and incubation

We used sterile swabs without a transport medium for sampling (Delta lab, Barcelona, Spain). The process was carried out on immediately after the hunting event. We placed the cotton swab into the nasal cavities of the animals. The collected samples were taken to the microbiology laboratory of the Kaposvár Campus of the Hungarian University of Agriculture and Life Sciences, where they were enriched in 5 ml of buffered peptone solution containing 10% sodium chloride. The relatively high salt concentration enhanced the reproduction of halophilic bacteria such as the members of the *Staphylococcus* genus. The incubation was carried out for 48 hours at 36 °C.

3.1.3 Identification and determination of phenotypic resistance

After incubation, the samples were spread on plate count agar (PCA). During this process, an inoculum was removed from the previously prepared and incubated bacterial suspension with a loop sterilized in flame. After spreading, the plate was repeatedly incubated at 36 °C for 24 hours.

At the end of the incubation period, the isolates were evaluated based on colony morphology. Gram staining was performed if homogeneous colony forms were observed on the agar. If the colony morphology was not uniform, a repeated spread was carried out from the different colonies to purify them. After passage, the pure isolates were incubated again at 36 °C for 24 hours. After their coherent formation in appearance, the isolates were judged by Gram staining. Using a light microscope, we evaluated the pure strains at 400x and 1000x magnification. We went on with the analysis of those samples, which proved Gram-positive cocci by light microscopy.

The species identification and phenotypic antibiogram determination of isolated strains were performed using a VITEK 2 Compact machine (bioMérieux, Crappone, France). The device is a semi-automatic system that analyses the growth of bacteria in different conditions. For identification, GP ID cards were used. Each card had 64 wells that contained a different substrate. Thus, using a card, it was possible to analyse 64 different biochemical processes during the growth of bacteria. Based on the reactions observed in each medium, the automata compared the results with a database. Based on their results, the equipment provided a prediction value for the sample species.

For the first step of the identification process, bacterial suspension was created, and the concentration of bacteria was adjusted. The inoculum was removed from the plate and placed in 3 ml of 0.45% saline solution. After thorough homogenization, the McFarland turbidity of the prepared suspension was measured. For this process, we used DensiCheck (bioMérieux, Crappone, France). We started the identification when the McFarland turbidity value was in the range of 0.5-0.63, as recommended by the manufacturer.

After inserting the cards, the analysis took approximately 5-8 hours at 35.5 °C. The equipment took colorimetric measurements in each card well every 15 minutes. It compared the results (colour changes during incubation) with an identification database, and finally gave a probabilistic result for the given sample. The identification was accepted if the probability level exceeded 85%.

P592 cards were used for antibiotic susceptibility testing. They were used to investigate phenotypic resistance to 17 antibiotics simultaneously. Each antibiotic (cefoxitin: CEF, benzylpenicillin: B-PEN, oxacillin: OXA, gentamicin: GEN, ciprofloxacin: CIP, moxifloxacin: MOX, erythromycin: ERY, clindamycin: CLI, linezolid: LIN, teicoplanin: TEI, vancomycin: VAN, tetracycline: TET, tigecycline: TIG, Fosfomycin: FOM, fusidic acid: FUS, rifampicin: RIF, trimethoprim: TRI) was present at three different concentrations.

In the first step, a suspension was prepared in 3 ml of 0.45% saline, as already described. A McFarland value of 0.5 to 0.63 was adjusted, and 270 ml of this suspension was pipetted into 3 ml of 0.45 % saline solution. After homogenization, it was inserted into the machine with the previously mentioned card. During the process, an inspection was also carried out in every 15 minutes, during which the equipment performed photometric measurements. As bacteria growth was expected in these cavities, the clarity of the starting suspension was constantly changing. The device used the minimum inhibitory concentration (MIC) to express the degree of resistance to each antibiotic.

3.1.4 Statistical analysis

The isolated bacterial strains were compared based on their bacterial similarity and the antibiotic resistance of the isolated *Staphylococcus* species. To express this, the Sørensen-Dice similarity index (SDI) was used (Dice, 1945; Sørensen, 1948):

$$SDI = \frac{2|X \cap Y|}{|X| + |Y|}$$

wherein

X = number of *Staphylococcus* species in a habitat or game species

Y = number of *Staphylococcus* species in the other habitat or game species.

The index value can be in the range 0-1. If the value is 1, then there is a complete similarity between the two habitats or hosts. A value of 0, on the other hand, means that no bacteria species or host in the studied habitats occurs in either place.

The occurrence of antimicrobial resistance is plotted on a map. The hunting area was divided into 2.5 kmx2.5 km UTM squares, and the prevalence of isolated resistant strains was

calculated for each quadrant. We also marked the places (settlements, livestock farms) on the map, with which the occurrence of resistance may be related.

3.2 Results and discussion

A total of 13 different *Staphylococcus* species samples were collected from 19 red deer and 19 wild boars. The ratio of isolated staphylococcus species in red deer and wild boar are shown in **Table 1** and **Figure 2**. *Staphylococcus aureus* was more prevalent in red deer while *S. scuri* was more prevalent in wild boar.

Table 1. *Proportion of staphylococcus species in red deer and wild boars*

species	red deer (n=19)	wild boar (n=19)
S. aureus	52.63%	0
S. carnosus ssp carnosus	0	4.34%
S. chromogenes	0	4.34%
S. equorum	5.26%	4.34%
S. gallinarum	0	8.7%
S. hominis ssp hominis	0	4.34%
S. hyicus	10.52%	17.04%
S. lentus	5.26%	0
S. saprophyticus	0	4.34%
S. sciuri	10.52%	21.74%
S. simulans	0	17.04%
S. warneri	5.26%	0
S. xylosus	10.52%	13.04%

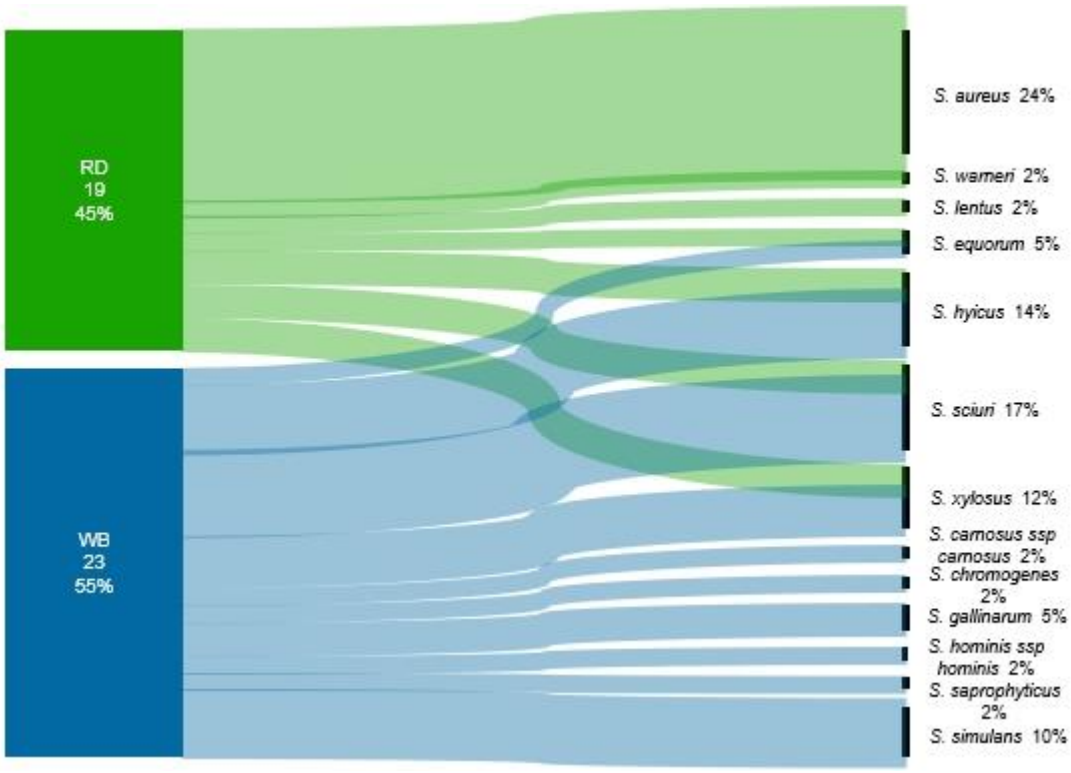

Figure 2. Summarised occurrence of isolated staphylococci

According to our findings, the prevalence of *Staphylococcus* species differed significantly across red deer and wild boar groups. Specifically, it was discovered that *Staphylococcus aureus* dominated in red deer, while *S. sciuri* dominated in wild boars. The prevalence of *S. aureus* in red deer agrees with the results of previous studies that found high rates of this species' staphylococcal carriage in populations of red deer that live freely. For instance, studies carried out in Germany and Spain reported transmission rates of 49% (Meyer et al., 2014) and 19.2% (Porrero et al., 2014), respectively, suggesting a significant *S. aureus* prevalence in red deer populations. Our results confirm this pattern, with more than half (52.6%) of the identified strains in red deer being *S. aureus*. This implies that red deer could act as environmental reservoirs for *S. aureus*, which could increase the risk of transmission to people and domestic animals.

On the contrary, *S. sciuri* was shown to be the most common species in wild boars. This result is in line with other research showing the frequency of *S. sciuri* in animals and environmental samples, especially among coagulase-negative staphylococci (CoNS) (Bonvegna et al., 2021). The prevalence of *S. sciuri* in wild boar's points to a microbial

community or environmental niche that is unique to this species and that facilitates the colonization and growth of this staphylococcal species.

The antibiotic susceptibility test, resulting in the *Staphylococcus* species sampled and cumulative proportion of resistance to each antibiotic in each host species, is shown in **Figure 3** and **Figure 4** respectively. My findings showed a significant resistance of *Staphylococcus* species to antibiotics especially in wild boar.

Most important antibiotic against which we found resistance was penicillin 16 of 41 strains (39.02%); four strains from RD (*S. aureus*: n=1, *S. sciuri*: n=2, *S. xylosus*: n=1) and 12 strains from WB (*S. sciuri*: n=6, *S. xylosus*: n=2, *S. gallinarium*: n=2, *S. saprophyticus*, n=1, *S. equorum*, n=1).

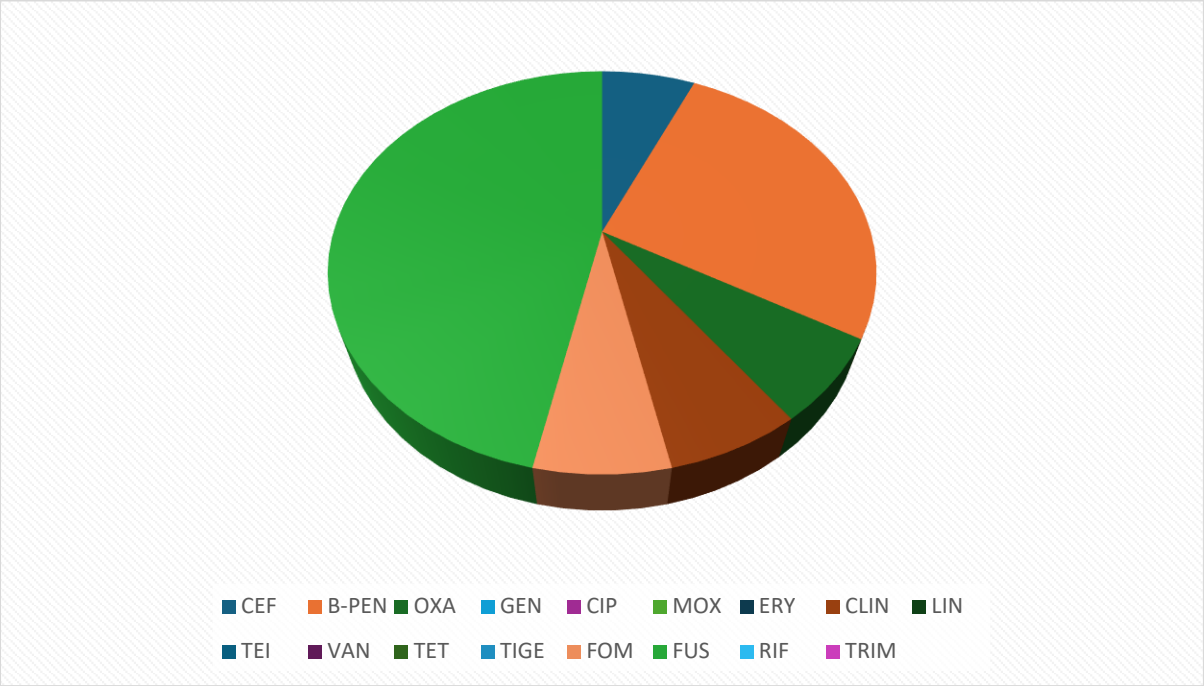

Figure 3. Proportion of resistance to antibiotics in red deer.

(Note: CEF-cefoxitin, B-PEN-benzylpenicillin, OXA-oxacillin, GEN-gentamycin, CIP-ciprofloxacin, MOX-moxifloxacin-ERY-erythromycin, CLIN-clindamycin, LIN-linezolid, TEI-teicoplanin, VAN-vancomycin, TET-tetracycline, TIGE-tigecycline, FOM-fosfomycin, FUS-fusidic acid, RIF-rifampicin, TRIM-trimethoprim)

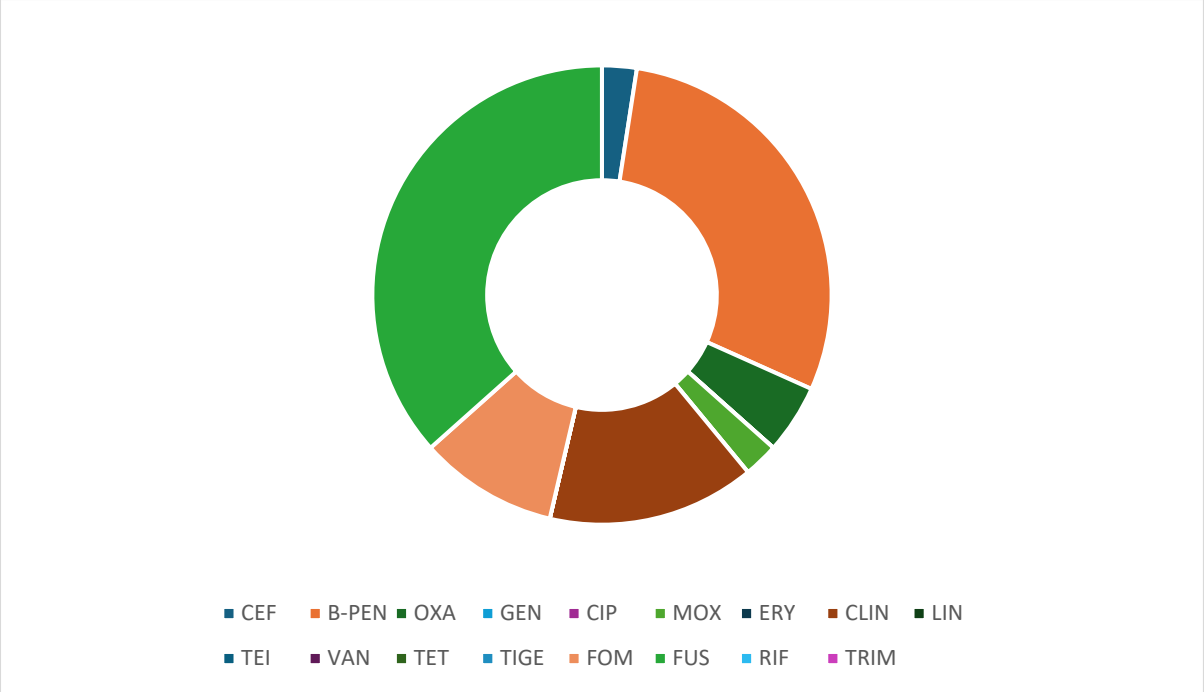

Figure 4. Proportion of resistance to antibiotics in wild boars.

(Note: CEF-cefoxitin, B-PEN-benzylpenicillin, OXA-oxacillin, GEN-gentamycin, CIP-ciprofloxacin, MOX-moxifloxacin-ERY-erythromycin, CLIN-clindamycin, LIN-linezolid, TEI-teicoplanin, VAN-vancomycin, TET-tetracycline, TIGE-tigecycline, FOM-fosfomycin, FUS-fusidic acid, RIF-rifampicin, TRIM-trimethoprim)

Table 2. The proportion of resistance in red deer and wild boars.

Antibiotics	Number of resistant strains (n=18) in red deer (proportion)	Number of resistant strains (n=23) in wild boar (proportion)
Cefoxitin	1 (5.56%)	1 (4.35%)
Benzylpenicillin	4 (22.22%)	12 (52.17%)
Oxacillin	1 (5.56%)	1 (4.35%)
Gentamicin	0	0
Ciprofloxacin	0	0
Moxifloxacin	0	1 (4.35%)
Erythromycin	0	0
Clindamycin	1 (5.56%)	6 (26.09%)
Linezolid	0	0
TEI	0	0

Vancomycin	0	0
Tetracycline	0	0
Tigecycline	0	0
Fosfomycin	1 (5.56%)	4 (17.4%)
Fusidic acid	7 (38.89%)	15 (65.22%)
Rifampicin	0	0
Trimethoprim	0	0

Our antibiotic susceptibility tests revealed concerning levels of resistance among the isolated *Staphylococcus* strains, particularly in wild boars. The cumulative proportion of resistance to each antibiotic was higher in wild boars compared to red deer. Of particular significance was the observation that 39.02% of strains exhibited resistance to penicillin, a critical antibiotic for both human and animal health. This aligns with existing literature suggesting a concerning level of antibiotic resistance in wildlife populations (Martínez-Seijas et al., 2023).

Based on the Sørensen-Dice similarity index, the staphylococci of the two studied host species showed a moderate overlap (SDI=0.471). The most important species for human infections, *S. aureus*, has only been found in red deer. Based on the occurrence of antibiotic resistance, red deer and wild boar overlapped markedly (SDI=0.833). Resistance was found to seven of the drugs tested (CEF, B-PEN, OXA, MOX, CLIN, FOM, FUS), of which moxifloxacin and clindamycin resistance were found only in wild boars.

Sixteen isolates (39.02%) showed resistance to penicillin, which has got great human and animal health concern. Four of these came from red deer and 12 from wild boars. The hunted games derived from six of the 27 UTM quadrants covering the study area. The incidence rate of penicillin resistance calculated on each square ranged from 20% to 100%. The highest value (100%) occurred in two quadrants, but only one strain was isolated from each of these. After the mapping (**Figure 5**), it was concluded that the human presence (settlement, tourist attraction, hotel) or significant livestock activity occurs in all concerned squares, which may have contributed to the spread of penicillin resistance into the environment.

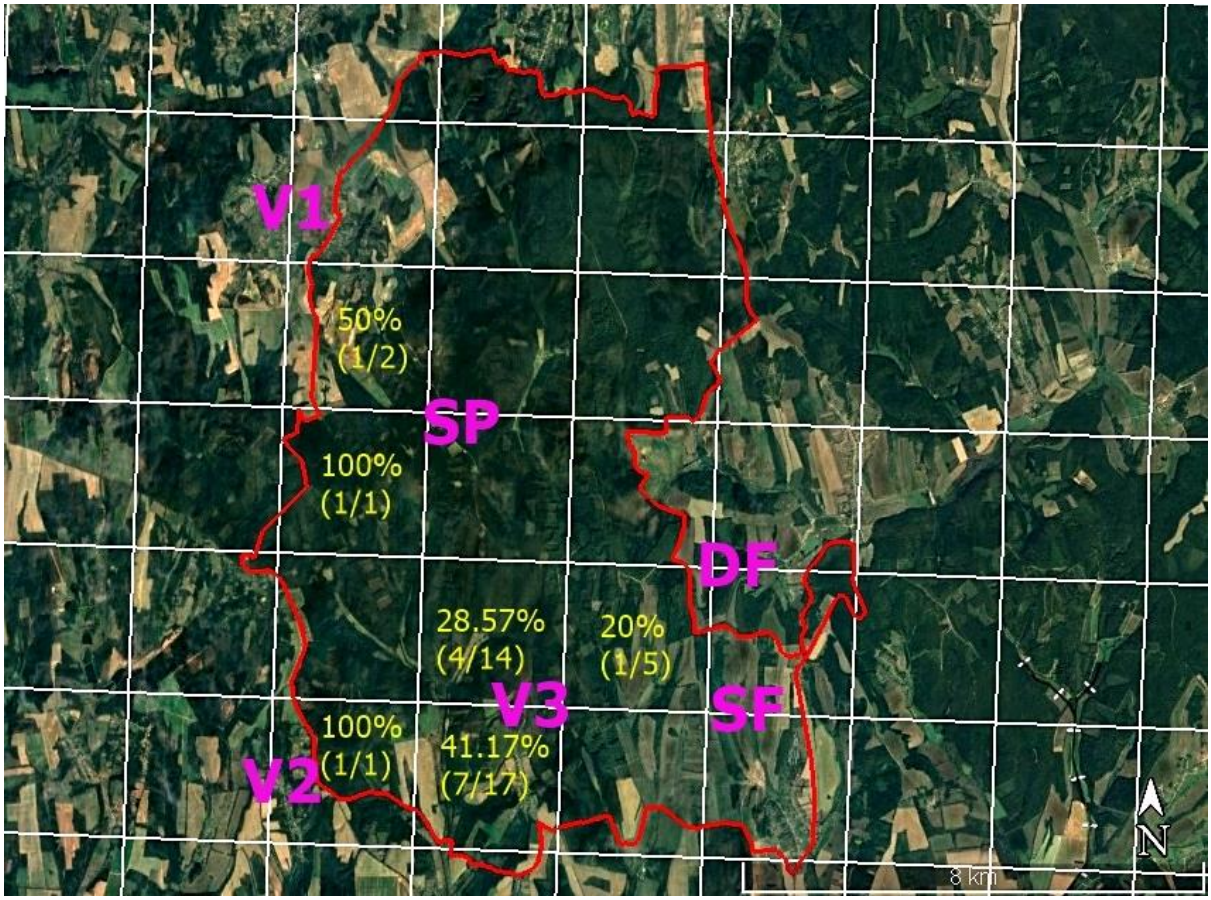

Figure 5. Mapping of penicillin resistance and the possible determinant factors.

(Note: V1, V2, V3= villages, SP: Zselic Starry Sky Park and Hotel Kardosfa – tourist attraction complex, DF: deer farm, SF: sheep farm).

There are concerns over the potential of resistant strains transmitting to people, domestic animals, and the environment due to the high prevalence of antibiotic resistance observed in wild boars. Additionally, the identification of antibiotic-resistant bacteria in wildlife habitats emphasizes the necessity of integrated One Health approaches to combat antimicrobial resistance because of the interconnected nature of human, animal, and environmental health. Our findings also demonstrate how crucial it is to consider how host ecology (contact with soil, water) and environmental factors can shape microbial communities and patterns of antibiotic resistance. For instance, the gut microbiota composition and antibiotic resistance susceptibility of red deer and wild boars may differ because of differences in their feeding environments.

Wild boars have a highly varied diet and can adapt to diverse habitats. As opportunistic omnivores, their dietary preferences are shaped by the availability of food items in their

environment, influencing their foraging behavior and population dynamics (Schley & Roper, 2003). Seasonal variations in the consumption of animal foods have been observed. For instance, studies by Briedermann (1976) and Tucak (1996) revealed that the consumption of small mammals peaked during autumn and winter, while earthworms were predominantly consumed in spring and summer, and molluscs in spring (Tucak, 1996). This suggests that plant and animal foods are not interchangeable. However, the relative consumption of animal material varies with age, with higher consumption observed in young boar (Dardaillon, 1984; Gérard et al., 1991; Wlaze ko & Abudzki, 1992; Abáigar, 1993; Groot Bruinderink et al., 1994). Their feeding behaviors may expose them to a broader range of microbes, potentially facilitating a more diverse microbiota that includes species like *S. sciuri,* which are less commonly associated with human infections but prevalent in environmental and animal reservoirs.

Red deer, categorized as intermediate ruminants, exhibit opportunistic feeding behavior by consuming a varied diet comprising grasses and concentrated food sources like browse, forbs, and fruits (Hoffmann, 1989). Their dietary preferences are shaped by the aim to maximize energy intake while minimizing the ingestion of potentially harmful compounds (Hanley, 1997). Red deer employ two main foraging strategies: concentrate selection, where they consume a diet rich in rapidly fermenting but less digestible materials, often containing tannins; or grazing, where they opt for slowly fermenting but more digestible fiber-rich diets with fewer anti-nutritional or toxic components (Demment & Van Soest, 1985; Verheyden-Tixier et al., 2008). In a different study, the findings affirmed the classification of red deer as intermediate feeders, with their diet consisting of a mixture of grasses and sedges (29%) alongside concentrate food items (63%). Despite this, red deer predominantly function as concentrate feeders, with concentrate food constituting up to 75% of their diet, and no significant seasonal fluctuations observed in the proportions of grasses/sedges versus concentrate food consumption (Gebert & Verheyden-Tixier, 2001).

The impact of human activities on wildlife behavior and habitat use cannot be overlooked (Coppes et al., 2017). Both red deer and wild boars exhibit responses to human disturbance, which may influence their exposure to environmental reservoirs of antibiotic-resistant bacteria. For instance, human recreational activities and habitat fragmentation may alter wildlife movement patterns, leading to increased contact with anthropogenic sources of antibiotic resistance. Studies have shown that red deer exhibit direct reactions to human

disturbance, including immediate flight, relocation to areas with dense vegetation cover, and temporary abandonment of disturbed areas (Miller et al., 2001). In regions with high levels of recreational activity, red deer may increase vigilance behaviors, potentially leading to decreased food intake. Ultimately, human disturbance may compel red deer to temporarily or permanently abandon optimal habitats, foraging instead in sub-optimal areas (Taylor & Knight, 2003).

On the other hand, continuous high human pressure tends to lead to a spatial shift in wild boar habitat use to avoid disturbed areas (Fischer et al., 2016; Tolon et al., 2009). Conversely, landscape variables such as habitat, food availability, and cover appear to modulate the magnitude of this response (Fattebert et al., 2017). In a study conducted to investigate how ungulates cope with human disturbance in a multi-use, suburban ecosystem, the results showed that the space use of wild boars does not seem to be impacted negatively by human activities, although responses may be hidden by the selection for cover in forest habitats providing seclusion from humans (Putman et al., 2019). Another study highlighted the opportunism of wild boar, suggesting that the local availability of forest habitat affects their ability to thrive in a human-dominated landscape characterized by intensive recreational use combined with fluctuating hunting pressure (Wevers et al., 2020

Antibiotic resistance patterns can be influenced by direct and indirect exposure to antimicrobial agents. While wild animals are not typically treated with antibiotics, they can encounter these compounds through environmental contamination such as water and soil polluted with residues from agriculture and pharmaceutical waste. Additionally, the interaction between wildlife, livestock, and pets can facilitate the horizontal gene transfer of ARGs across different bacterial populations, further disseminating resistance (Landers et al., 2012). The same study also revealed the intricate web of interactions characterizing the farm environment. This includes the relationships among animals on the farm, interactions between farmers and animals, as well as connections between domestic animals and wildlife. Such interactions extend through a variety of farm activities, such as daily tasks like milking or grazing, and encompass aspects like storage and food preparation. Moreover, these dynamics interact with the physical environment including air, soil, and water; and are influenced by human behaviour patterns, including food processing, consumption habits, and vulnerability to infections (Landers et al., 2012).

Misuse of antibiotics in livestock can lead to antibiotic resistance, a concern with long-standing evidence and potential implications for human health (Landers et al., 2012). In livestock, antibiotics serve not only to treat illnesses but also to prevent infections, such as using them prophylactically in female animals following difficult births to avert post-partum endometritis. Despite the critical nature of antibiotic usage patterns in animals, global data on this subject remains limited (Sarmah et al., 2006). The practice of administering antibiotics to newborn livestock as a preventative measure against neonatal infections is particularly problematic. This approach significantly contributes to the emergence of antibiotic-resistant bacteria on farms and should be avoided (Lianou & Fthenakis, 2022).

Regulatory measures and periodic residue testing in animal products are implemented to encourage farmers to adhere to antibiotic withdrawal periods. However, a study in Greece found antibiotic residues in 12.1% of sheep milk samples (Durazzo et al., 2022), indicating a discrepancy between the observance of withdrawal periods and the detection of residues. This suggests that despite efforts to comply with withdrawal guidelines, errors in antibiotic application, such as inaccurate dosage related to the animal's weight, may still lead to the presence of residues in milk.

3.3 Conclusions and suggestions

The study was carried out on red deer (n=19) and wild boars (n=19) hunted in a natural habitat. During the study, we sought to answer which species of staphylococci occur in the two host species and to which antibiotics they show resistance. Microbiological studies showed a moderately strong overlap between bacterial species in red deer and wild boar (SDI=0.5). The most common species in the red deer was *S. aureus* (52.61%). While in wild boar, *S. sciuri* proved the most prevalent bacterial species (21.74%).

The antibiograms of 42 isolated strains were examined (red deer: n=19; wild boar: n=23). Penicillin resistance was observed in 39.02% of all isolates: four of the 18 red deer-originated strains were resistant (21.05%), while 12 from the 23 wild boar-originated samples were resistant (52.17%). In total, two strains showed methicillin resistance one, a *S. xylosus*, was isolated from red deer and another one, a *S. equorum,* was isolated from wild boar.

During the spatial analysis of the samples, it was observed that some penicillin-resistant isolates were more likely to occur near settlements or touristic attractions frequently visited by people. The other part of the resistant strains was found in habitats near intensive livestock farming.

The findings highlight that resistance to some antibiotics is widespread in wild populations of natural habitats where no medication has been taken. The high level of penicillin resistance in staphylococci from the tested host species demonstrates that even bacterial species and their genes can easily appear and spread between environmental, livestock and human domains.

There was a significant difference in the proportion of resistant isolates between red deer and wild boar. One reason may be the species' different nutrition. Red deer typically consume only plant material. Consequently, the uptake of resistant bacteria is conceivable on pastures and artificial feeders for wildlife that are also intensively used by other species (e.g., sheep, beef cattle, wild boar). The wild boar is an omnivore species; components of animal origin (e.g. earthworms) may appear in its diet in significant quantities. As a result, it can also intake higher amounts of resistant bacteria that may accumulate in the food chain or the soil.

The two species are characteristically different in their tolerance to humans. Red deer avoid areas where human presence is high. It responds to continuous interference by avoiding space or time. Consequently, the risk of direct infection with resistant bacteria associated with human presence is low. Wild boar, on the contrary, tolerates the presence of human better. Sometimes, that increased human presence and its consequences are beneficial. In the habitat used by wild boars, littering can even serve as a food source. Thus, direct uptake of resistant pathogens transmitted by human waste may occur more often.

In the context of the findings, it may be recommended that wild species hunting bags in certain natural habitats should be monitored for resistant bacteria. The monitoring could be done very effectively in game processing plants, where the shot game comes from all areas of the country. Considering the results, these control tests should also be carried out on wild boar carcasses since resistant strains can be better traced in this species. Another reason for including the species is that deer carcasses arrive at processing plants without their heads. Due to this, the examination of bodies may lead to low efficiency. Since boar carcasses are

processed together with their heads, it is possible to sample from the nasal and oral cavities. As a result, the effectiveness of the test may also be much higher than in the case of cervids.

Based on the results, we concluded that resistance to antibiotics is present even in natural habitats. Its spread between the domains of environment, animals, and humans poses significant health and economic risks. Therefore, it is advisable to develop surveillance programmes within which different habitats are continuously monitored. Only with such comprehensive studies can control strategies be developed to slow the spread of resistant bacteria.

4. SUMMARY

This study aimed to collect data on the occurrence and antibiogram of the *Spathylococcus* genus members in a natural habitat shared with different game species. The investigation was carried out on red deer (n=19) and wild boars (n=19) hunted in natural habitat located in southwestern Hungary. A total of 42 strains of *Staphylococcus* strain were isolated, of which 18 were red deer origin, and 23 were wild boar origin. The number of species found in both host species was four (*S. equorum*, *S. hyicus*, *S. sciu*ri, and *S. xylosus*). In addition, two more species were isolated exclusively from red deer (S. aureus, S. lentus) and six more from wild boar (*S. carnosu*s ssp. *carnosus*, *S. chromogenes*, *S. gallinarum*, *S. homisi* ssp. *hominis*, S. *saprophyticus*, *S. simulans*). The most prevalent germs were *Staphylococcus aureus* (52.63%) in red deer and *S. scuri* (21.74%) in wild boar.

All but one isolate could be subjected to antibiograms. The analysis revealed that 16 isolates showed penicillin resistance (39.02%). One isolate from both wild boar and red deer was also found to be methicillin resistant.

Based on the Sørensen-Dice similarity index, the staphylococci of the two studied host species showed a moderate overlap (SDI=0.471). Regarding antibiotic resistance, staphylococci isolated from the two species showed a strong closeness (SDI=0.833). Based on the localisation of the animals carried penicillin-resistant strain, it was concluded that the human presence (settlement, tourist attraction, hotel) or significant livestock activity occurs in all concerned localities, which may have contributed to the spread of penicillin resistance in the environment. The difference in the proportion of resistant isolates between red deer and wild boar may be explained by two reasons. The first is the species' different nutrition. Red deer typically consume only plant material. Consequently, the uptake of resistant bacteria is conceivable on pastures and artificial feeders for wildlife that are also intensively used by other species (e.g., sheep, beef cattle, wild boar). The wild boar is an omnivore species with a high rooting and scavenging activity; therefore, this host may accumulate resistant bacteria from the food chain or the soil. Secondly, the two species are characteristically different in their tolerance to humans. Red deer avoid areas where human presence is high. It responds to continuous interference by avoiding in space or time. Consequently, the risk of direct infection with resistant bacteria associated with human presence is low. Wild boar, on the contrary, tolerates better the presence of human. Thus, direct uptake of resistant pathogens transmitted by human waste may occur more often.

Based on the results, we concluded that resistance to antibiotics is present even in natural habitats. Its spread between the three domains of health, environment, animals, and humans, poses significant health and economic risks. Therefore, it is advisable to develop surveillance programmes within which different habitats should be continuously monitored. Only with such comprehensive studies control strategies can be developed to slow the spread of resistant bacteria.

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