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Antimicrobial resistance in commensal Staphylococcus aureus from wild ungulates is driven by agricultural land cover and livestock farming

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Abstract:	Staphylococcus aureus is a human pathobiont, a nosocomial pathogen and a leading cause of morbidity and mortality in humans. S. aureus is also a pathobiont of companion animals and livestock. The dissemination of antimicrobial resistant (AMR) S. aureus , particularly methicillin-resistant (MRSA), has been associated to its ability for establishing new reservoirs, but limited attention has been devoted to the environment. To fill this gap, we aimed to characterize animal carrier status, AMR phenotypes, predominant clonal lineages and their relationship with clinical and food-chain settings, as well as to find predictors of AMR occurrence. Nasal swabs (n =254) from wild boar (n =177), red deer (n =54) and fallow deer (n =23) hunted in Portugal, during 2019/2020, yielded an overall carrier proportion of 35.8%, ranging from 53.7% for red deer and 32.2% for wild boar to 21.7% for fallow deer. MRSA from wild boar and phenotypically linezolid-resistant S. aureus from wild boar and red deer were isolated, indicating that resistance to antimicrobials restricted to clinical practice also occurs in wildlife. The most prevalent genotypes were t11502/ST2678 (29.6%) and t12939/ST2678 (9.4%), previously reported in wild boar from Spain. Clonal lineages reported in humans and livestock, like CC1, CC5 or CC8 (19.1%) and ST425, CC133 or CC398 (23.5%), respectively, were also found. The sequence type ST544, previously restricted to humans, is described in wildlife for the first time. We also identified that land use (agricultural land cover), human driven disturbance (swine abundance) and host-related factors (sex) determine resistance occurrence. These findings suggest that antibiotics used in clinical settings, agriculture and livestock farming, spill over to wildlife, leading to AMR emergence, with potential ecological and human health effects. This is one of the most comprehensive surveys in Europe of S. aureus occurrence and determinants among widely distributed wild ungulates.
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Response to Reviewers:	

Dear Editor of Environmental Pollution,

please find herewith the research article "**Antimicrobial resistance in commensal *Staphylococcus aureus* from wild ungulates is driven by agricultural land cover and livestock farming**".

Staphylococcus aureus is a human pathobiont, a nosocomial pathogen and a leading cause of morbidity and mortality, whose infection success has been associated with its ability for establishing new reservoirs. Contaminated environments or environmental pollution that derive from human activities and human practices in livestock husbandry have been pointed out as important drivers of antimicrobial-resistant bacteria spill-over, but solid evidence remains lacking. Also, to the best of our knowledge, detailed studies linking ecological determinants with antimicrobial resistance at the human-animal-environment interface remain very scarce. In this work, we use *S. aureus* as a bacterial model for a pathobiont and wild ungulates as animal model species to determine the impact of biotic and abiotic factors on antimicrobial resistance occurrence. Wild ungulates may establish the link between the transmission routes of antibiotic resistance determinants and/or bacteria from different sources (e.g. urban, natural and semi-natural) through a complex web of interactions, as they have large home ranges, are unlikely of being treated with antibiotics, and their habitat can overlap with livestock and humans. Furthermore, distinctive differences among these species across their ecological features (e.g. feeding habits, space use) and life history traits makes them good models to assess the influence of anthropogenic-associated factors in AMR and bacterial spread. With this work, we thus aimed to 1) characterize the animal carrier status for *S. aureus*; 2) describe the molecular types and antimicrobial resistance profiles of bacteria isolated from sampled hosts; 3) investigate the predominant clonal lineages and their relationship with clinical and food-chain settings; and 4) determine the impact of biotic and abiotic factors on AMR occurrence. Nasal swabs (n=254) from hunter harvested wild boar (n=177), red deer (n=54) and fallow deer (n=23) were screened for *S. aureus*, in one of the most comprehensive surveys in Europe, yielding an overall carrier proportion of 35.83%. Through molecular typing, we were able to associate over one third of the retrieved *S. aureus* isolates to both humans and livestock species, as well as to identify resistance to high-end treatment antimicrobials like methicillin and linezolid. The impact of environmental pollution related with specific human practices (land use, animal husbandry and management) could be indisputably linked to the occurrence of antimicrobial resistance through ecological modelling.

This work highlights the need to restrict the sources of environmental contamination with xenobiotics and AMR bacteria, namely those linked to human disturbance and landscape conversion towards agricultural lands. Simultaneously, we reinforce the significance of maintaining a comprehensive approach under the realm of One Health by dissecting the several components at the human, environment and livestock interfaces that may act upon the lifecycle of antimicrobials and AMR bacteria.

We hereby declare that the manuscript content has not been published or submitted for publication elsewhere.

All enlisted authors have contributed to this study and they all are in agreement with the content of the manuscript.

We believe that this research article meets high-quality research standards, deserving wide dissemination to the community that will be warranted by publication in Environmental Pollution.

Yours sincerely,

Mónica V. Cunha (on behalf of all authors)

Prof. Sarah Michele Harmon
Editor, Environmental Pollution

Lisboa, 02nd of March 2022

Re: Antimicrobial resistance in commensal *Staphylococcus aureus* from wild ungulates is driven by agricultural land cover and livestock farming (Manuscript ID: ENVPOL-D-21-07332-R1).

We are grateful for the constructive comments made by peers on this work and for the editorial opportunity to revise the manuscript. Following the comments and suggestions of the referees, we have made improvements to our original submission. We carefully addressed reviewers' concerns and included the comments/suggestions in the revised version of the manuscript. Below we provide a detailed outline of how this manuscript has been revised in light of editor and referees' comments. We do believe the current draft is complete and we hope you will now judge it acceptable.

Kind regards,

Mónica V. Cunha (PhD), on behalf of all authors

Responses to Referees comments:

Reviewer #2:

I am satisfied with the revision done by the author(s).

To improve the final document, a few minor comments underlined in yellow are highlighted on the revised PDF document, suggesting occasional point changes.

Author's response: All the occasional point changes were addressed along the manuscript, and some specific comments are addressed more fully in the next responses.

Line 382: MIC=4 is not resistant. Do not consider as resistant.

Author's response: The authors highlight in lines 371-373 that this specific isolate, although susceptible, stands at the MIC breakpoint which might be a matter of concern for hypothetical gain of resistance. However, in lines 377-378 it is referred that "In the whole set of 303 isolates, **resistance to either trimethoprim-sulfamethoxazole or vancomycin was not detected**.", meaning that none of the retrieved isolates were considered vancomycin resistant.

Line 553: xenobiotics in feed additives for livestock? Which?

Author's response: The use of antibiotics for metaphylactic purposes, e.g., aminoglycosides, polymyxins, penicillins and tetracyclines, in livestock farming (Pokludová, L., 2020), is pointed to be a driver of AMR development through human disturbance.

Highlights

- We report results from a broad survey of pathobiont *S. aureus* among wild ungulates.
- The overall carrier status was 35.83% (95%CI: 29.89-41.76%), peaking in red deer.
- Clonal lineages from wild ungulates were mostly associated to humans or livestock.
- Resistance to antimicrobials restricted to clinical practice was found in the wild.
- Land use, livestock abundance and host traits determine AMR occurrence.

graphical abstract

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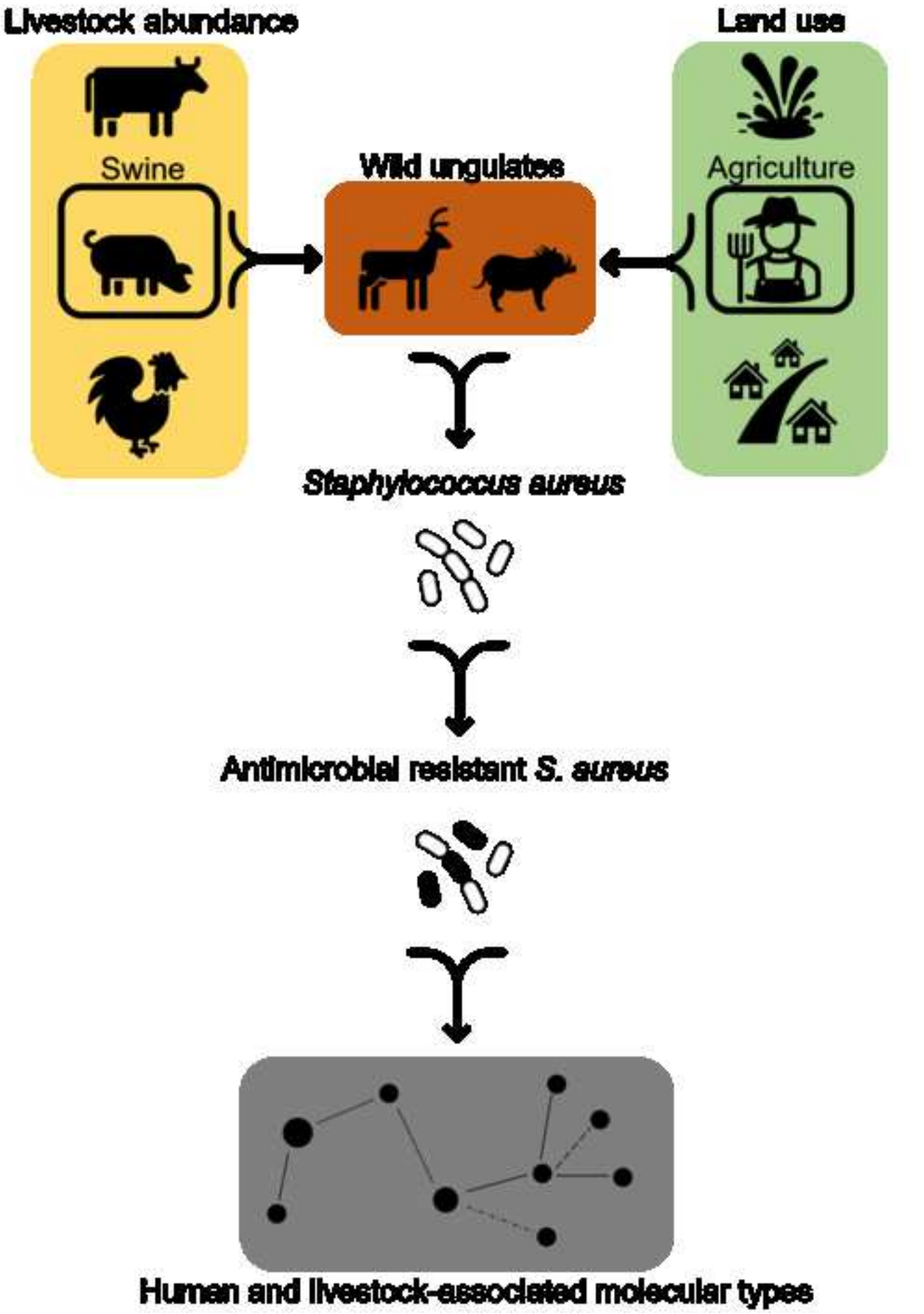

Antimicrobial resistance in commensal *Staphylococcus aureus* from wild ungulates is driven by agricultural land cover and livestock farming

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Keywords: *Staphylococcus aureus*; MRSA; MLST; wildlife; antimicrobial resistance; ecological modelling; human perturbation.

ABSTRACT

Staphylococcus aureus is a human pathobiont (i.e., a commensal microorganism that is potentially pathogenic under certain conditions), a nosocomial pathogen and a leading cause of morbidity and mortality in humans. *S. aureus* is also a commensal and pathogen of companion animals and livestock. The dissemination of antimicrobial resistant (AMR) *S. aureus*, particularly methicillin-resistant (MRSA), has been associated to its ability for establishing new reservoirs, but limited attention has been devoted to the role of the environment. To fill this gap, we aimed to characterize animal carrier status, AMR phenotypes, predominant clonal lineages and their relationship with clinical and food-chain settings, as well as to find predictors of AMR occurrence. Nasal swabs (*n*=254) from wild boar (*n*=177), red deer (*n*=54) and fallow deer (*n*=23) hunted in Portugal, during the season 2019/2020, yielded an overall carrier proportion of 35.8%, ranging from 53.7% for red deer and 32.2% for wild boar to 21.7% for fallow deer. MRSA from wild boar and phenotypically linezolid-resistant *S. aureus* from wild boar and red deer were isolated, indicating that resistance to antimicrobials restricted to clinical practice also occurs in wildlife. The most prevalent genotypes were t11502/ST2678 (29.6%) and t12939/ST2678 (9.4%), previously reported in wild boar from Spain. Clonal lineages reported in humans and livestock, like CC1, CC5 or CC8 (19.1%) and ST425, CC133 or CC398 (23.5%), respectively, were also found. The sequence type ST544, previously restricted to humans, is described in wildlife for the first time. We also identified that land use (agricultural land cover), human driven disturbance (swine abundance) and host-related factors (sex) determine resistance occurrence. These findings suggest that antibiotics used in clinical settings, agriculture and livestock farming, spill over to wildlife, leading to AMR emergence, with potential biological, ecological, and human health effects. This work is one of the most comprehensive surveys in Europe of *S. aureus* occurrence and determinants among widely distributed wild ungulates.

INTRODUCTION

Staphylococcus aureus is a pathobiont (i.e., a potentially pathogenic microorganism under certain circumstances), colonizing the nasal mucosa and skin of humans and, often, other animals, e.g., swine (Strube, Hansen, Rasmussen, & Pedersen, 2018). However, it is also associated to human disease, from skin or soft tissue infections to systemic and fatal illness (Sakr et al., 2018). While *S. aureus* has been most commonly reported as a human and nosocomial pathogen, it has been also isolated from a wide range of wild species, mainly mammals and birds (Feßler et al., 2018; Schaumburg et al., 2012), in recent years. Additionally, it has been reported in environmental matrices, such as water and wastewater (Silva et al., 2020), air (Kozajda, Jeżak, & Kapsa, 2019), and soil (Johny, 2019).

The role of non-human hosts in *S. aureus* transmission is emphasized by the fact that most clonal complexes (CCs) include strains recovered from diverse ecological settings (Matuszewska et al., 2020). A good example is the case of antimicrobial resistant (AMR) *S. aureus*, particularly methicillin-resistant (MRSA), which have acquired resistance to β-lactams, posing major public health concerns. MRSA have been recovered from numerous healthy and sick animal species, including livestock, companion animals (Monecke et al., 2016) and wildlife (Porrero, Mentaberre, et al., 2014; Ruiz-Ripa et al., 2019; Seinige, Von Altrock, & Kehrenberg, 2017). Dissemination of AMR *S. aureus* has been associated to its ability for establishing new reservoirs (Heaton et al., 2020). The results obtained from molecular characterization has allowed to link particular CCs, like CC398 or CC133, to the animal component, namely livestock, on *S. aureus* transmission (Hoekstra et al., 2020; Price et al., 2012). However, typical human CCs, like CC1, CC5 or CC8, have also been reported in livestock, raising concerns on host jumps (Heaton et al., 2020; Richardson et al., 2018).

Wildlife contact with xenobiotics and antimicrobial agents can happen either by indirect contact with contaminated environmental matrices or environmental pollution driven by

livestock farming and human activity, or from direct contact with livestock in aggregation points during feeding (Torres, Carvalho, et al., 2020). Animals colonized with AMR bacteria may spill-over bacteria or antimicrobial resistance genes (ARG) to conspecifics and other species (Plaza-Rodríguez et al., 2021; Torres, Fernandes, et al., 2020; Vittecoq et al., 2016). In recent years, reporting AMR isolates in wildlife is increasingly common (Monecke et al., 2013; Palmeira et al., 2021; Porrero, Mentaberre, et al., 2014; Ruiz-Ripa et al., 2019; Torres, Fernandes, et al., 2020). This fact might be justified, not only by an increasing interest in wildlife health research, but also by the increase of interspecific transmissions in the human-livestock-wildlife interface (Torres, Carvalho, Cunha, & Fonseca, 2019). Prophylactic and metaphylactic treatments of farmed animals are considered the main drivers of antimicrobial resistance in bacteria (Marshall & Levy, 2011). The intensive use of antimicrobials in agriculture or aquaculture that promote environmental contamination also exert downstream selective pressure upon microorganisms (Laxminarayan et al., 2013).

In Portugal, antimicrobial resistant *Staphylococcus* spp., or typically healthcare-associated strains (Nazareth et al., 2012; Peres, Pina, & Fonseca Cardoso, 2011), and MRSA, have been isolated from pet dogs (Silva, Oliveira, et al., 2021), wild rabbits (Sousa et al., 2020), wild rodents (Silva, Gabriel, et al., 2021) and wild boars (Sousa et al., 2017). Detection of *mecA* or *mecC* has also been reported (Silva, Gabriel, et al., 2021; Sousa et al., 2017). The molecular characterization of wildlife isolates indicates the circulation of strains from diverse CCs, such as CC1, CC5, CC8, or the livestock-associated CC133 or CC398 strains (Silva, Gabriel, et al., 2021; Sousa et al., 2017; Sousa et al., 2020). Particular attention has been given to clonal lineages widely spread in humans, livestock and companion animals, but information on the incidence of MRSA in the environment, particularly in wildlife, remains overlooked.

In this work, we aimed to extend previous knowledge on the occurrence of *S. aureus* in wildlife, namely in three species of wild ungulates, such as wild boar (*Sus scrofa*), red deer (*Cervus*

elaphus) and fallow deer (*Dama dama*), which present contrasting ecology (e.g. feeding and space use strategies) and degree of synanthrophization (Torres, Fernandes, et al., 2020). We also aimed to disclose the drivers contributing for antimicrobial resistance among commensal bacteria or pathobionts from wild species that have limited or indirect contact with anthropogenic sources of antimicrobials (Torres, Fernandes, et al., 2020). For this purpose, we used *S. aureus* as a bacterial model, due to its ubiquity and wild ungulates as animal model species. Indeed, these species are affected by several factors that have impact their distribution and the way they use space, with repercussions in their ability to move and disperse AMR determinants. The specific aims of this work are: 1) to characterize the animal carrier status for *S. aureus*; 2) to describe the molecular types and antimicrobial resistance profiles of *S. aureus* isolated from sampled hosts; 3) to investigate the predominant clonal lineages and their relationship with clinical and food-chain settings; and 4) to determine the impact of biotic and abiotic factors on the occurrence of AMR *S. aureus*.

These aims could be reached by means of a large survey across different environmental contexts, taking advantage of biological specimen availability from hunted animals and ecological modelling, setting the scene for one of the most comprehensive studies in Europe of *S. aureus* occurrence among widely distributed wild ungulates, at the frontier of humanized landscapes.

METHODS

Sample collection

A total of 254 nasal swabs from three species of wild ungulates (wild boar, *n*=177; red deer, *n*=54; fallow deer, *n*=23) were collected during the 2019/2020 legal hunting season (from October 2019 to February 2020) across mainland Portugal. Animals were sampled, and nasal specimens were collected within 1-5 h after death. Nineteen different sampling sites were

included in this study (Supplementary Figure 1), encompassing a high diversity of land cover (illustrated in Supplementary Figure 2) and also capturing different animal densities. Host species, sex and age class were registered for each sampled animal.

Selective isolation of *Staphylococcus aureus*

For selective isolation, all nasal swabs were subjected to selective enrichment in Mueller-Hinton medium (Sigma, Merck KGaA, Germany) supplemented with 6.5% NaCl, to select for the characteristic halotolerance of clinically-relevant *Staphylococcus* sp., namely *S. aureus*. After 18-24 hours incubation at 35ºC±1ºC, each pre-enriched sample was plated (100 µl) onto Mannitol Salt Agar (MSA) (Oxoid, Thermo Fisher Scientific, USA) and colonies suggesting mannitol fermentation, and thus hypothesized as *S. aureus*, were selected from each plate. Up to eight colonies with distinctive morphology or size from each plate, grown from each enrichment in MSA, were sub-cultured onto Nutrient Broth Agar (Biokar, Solabia, France). All isolates retrieved from MSA were tested for β-haemolysis in Columbia agar medium with 5% sheep blood (bioMérieux Clinical Diagnostics, France). The previous protocol was adapted from the procedure described in "Isolation of methicillin-resistant *Staphylococcus aureus* (MRSA) from food-producing animals and farm environment" from the European Food Safety Authority (EFSA) (European Food Safety Authority, 2012). A quality control strain (*Staphylococcus aureus* ATCC 25923) was used to interpret and validate each test batch.

All isolates that tested positive for β-haemolysis were subjected to molecular identification as *S. aureus* through polymerase chain reaction (PCR), performed according to Martineau and collaborators (Martineau et al., 1998), by means of two *S. aureus*-specific primers and an additional set of 16S rRNA primers for internal amplification control (Marchesi et al., 1998). The full characterization of each *S. aureus* isolate is described in Supplementary Table 1.

Molecular amplification of *spa* hypervariable region and Multi-Locus Sequencing Typing

149 The molecular amplification of the *spa* hypervariable region was performed by PCR using the
150 primers described by Stegger and collaborators (Stegger et al., 2012). Multi-locus sequencing
151 typing (MLST) (Enright et al., 2000) was performed for a single random isolate representative
152 of each retrieved *spa* type, comprising a total of 20 isolates representing 19 *spa* types. Strain
153 *Staphylococcus aureus* ATCC 25923 was used as positive control in all PCR assays. A no-
154 template control with nuclease-free water was also added in each PCR assay.

155 The *spa* amplicons and MLST fragments were sequenced and assigned using *spa* typing and
156 MLST plugins, respectively, from BioNumerics v6.6 (Applied Maths).

157 The discriminatory power (D) of each technique, i.e. the average probability that the typing
158 method will assign a different type to two unrelated isolates sampled in the population, was
159 calculated based on Simpson's index of diversity (Hunter & Gaston, 1988) using the online tool
160 "*In silico* simulation of molecular biology experiments" (Bikandi, Millán, Rementeria, &
161 Garaizar, 2004). The individual allelic diversity (h) of the *loci* from MLST scheme tested were
162 obtained in a similar manner.

163 **Phylogenetic analysis**

164 For the phylogenetic analysis, one minimum spanning tree was computed with results from
165 *spa* typing as input, using BioNumerics v6.6 (Applied Maths). The advanced cluster analysis
166 was performed using the categorical data as a similarity coefficient, and calculated a standard
167 minimum spanning tree with single *locus* and double *loci* variance priority rule. The
168 dendrogram was computed using *spa* fragment sequences as input using BioNumerics v6.6
169 (Applied Maths) calculating Pearson correlation coefficient, and the unweighted pair group
170 method with arithmetic means (UPGMA) as the agglomerative clustering algorithm. The
171 annotation was performed using Interactive Tree Of Life (iTOL) v5 (Letunic & Bork, 2021).

172 **Antimicrobial susceptibility testing**

Antimicrobial susceptibility testing was performed by disk-diffusion and broth microdilution methods, according to the guidelines of the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2021). The following antimicrobial agents were tested by disk-diffusion (µg per disk): benzylpenicillin (1 unit), cefoxitin (30), ciprofloxacin (5), erythromycin (15), gentamicin (10), tetracycline (30), linezolid (30) and trimethoprim-sulfamethoxazole (1.25 + 23.75). The broth microdilution assays were performed for oxacillin and vancomycin, according to EUCAST guidelines (EUCAST, 2021). Briefly, a McFarland 0.5 suspension of all isolates was used to inoculate Mueller-Hinton broth or Mueller-Hinton Agar (Oxoid, Thermo Fisher Scientific, USA), for broth microdilution and disk diffusion methods, respectively, and incubated overnight at 37°C. This panel of antimicrobials represent eight antimicrobial classes: aminoglycosides (gentamicin), β-lactams (oxacillin, cefoxitin and benzylpenicillin), fluoroquinolones (ciprofloxacin), folate pathway inhibitors (trimethoprim-sulfamethoxazole), glycopeptides (vancomycin), macrolides (erythromycin), oxazolidinones (linezolid) and tetracyclines (tetracycline), and thus the most frequent cellular targets, namely cell envelope, protein synthesis at both 30S and 50S ribosomal subunits and nucleic acids biosynthesis. All antimicrobial susceptibility tests were evaluated according to EUCAST clinical criteria, as we intended to link the retrieved isolates with the potential spill-over of human-associated isolates into the environment. The genotypic resistance profile to β-lactams was evaluated based on the amplification of *mecA* and *mecC* gene markers, according to Stegger and colaborators (Stegger et al., 2012). Multidrug resistant isolates were defined according to Magiorakos and co-workers (Magiorakos et al., 2012): resistance to at least one antimicrobial from at least three different antimicrobial classes or identification as MRSA through molecular detection of either *mecA* or *mecC* gene markers.

Statistical analyses

Statistical analyses were performed using non-parametric Mann-Whitney test for independent and continuous variables in order to evaluate differences in carrier proportion across host, sex, and age. The *p* values were determined and discussed accordingly using α=0.05 to reject the null hypothesis (considered significant if $P < 0.05$). The 95% confidence intervals (CI) were also estimated. Multivariate analyses, namely Principal Components Analysis (PCA), were performed for the top five most frequent molecular types and for all classes of resistance phenotypes (i.e. multidrug resistant, resistant and susceptible), using "prcomp" function from R software v4.0.5 and the threshold established for relevant association was ±0.2. The exploratory approach was performed with the following variables: sampling district (Beja, Évora, Portalegre and Setúbal [Alentejo], Coimbra, Leiria, Guarda [Central Portugal], Porto [North Portugal]); sex (male and female); age (adult and juvenile); host species (wild boar, red deer and fallow deer).

Geographic information was used at different scale levels (NUTS II statistical regions, district administrative region, municipality administrative region) whenever appropriate.

Modelling procedures

Having in mind the ecology of wild ungulates and the factors that may facilitate exposure to antimicrobials, and thus impact the emergence of AMR bacteria and/or the exchange of AMR determinants, we explored the effect of environmental, host-related, land use, human driven disturbance factors or spill-over from livestock on AMR occurrence. To test these hypotheses, we examined 17 variables as putative predictors of AMR occurrence. For this analysis, six categories of variables were considered, representing different working hypothesis, as described in Table 1. All variables were considered at the Municipality level due to the lack of the exact geographical coordinates of the location where each animal was hunted. Land use data was retrieved from Carta de Uso e Ocupação do Solo (2018) at the National Geographic Information System [Sistema Nacional de Informação Geográfica (SNIG)] (Direção Geral do

Território - Portugal, 2018). Livestock abundance (2019) (INE, 2019) and population density (2020) (INE, 2020) were gathered from Instituto Nacional de Estatística (INE). Wild ungulates presence was defined based on the Atlas of Mammals in Portugal (Bencatel J. et al., 2019). Climate data comprised variables gathered from BioClim (2017) at 30 arc-second resolution (Fick & Hijmans, 2017). Both host sex and age class data were encoded as binary, where female and juvenile are encoded by zero and male and adult are encoded by one.

Regarding the modelling procedure, initially the data was tested for spatial autocorrelation to account for the degree of the geographical dependency of data, using the Moran's I index with the R's "ape" package (Paradis & Schliep, 2018), using the municipality centroid as a surrogate of the animal's hunting location. Multicollinearity was tested by estimating the Variation Inflation Factor (VIF) between variables within the same hypothesis, using R's "corvif" function from source code "HighstatLibV10" (Zuur, Hilbe, & Ieno, 2013). Variables with an estimated VIF above five were discarded from analysis, being that value considered a cut-off for excessive inflation in variance caused by the existing multicollinearity (Zuur et al., 2013). All the remaining candidate variables were standardized prior to modelling procedure for straightforward results' analysis. We applied a Generalized Linear Mixed Model (GLMM) approach to identify the drivers shaping antimicrobial resistance presence/absence variation. GLMM were built using a logit link function applied to a binomial distribution, and considering municipality as a random effect due to the presence of spatial autocorrelation of data (F. Dormann et al., 2007). Such modelling procedures were applied using the "lme4" package in R v.4.0.5 (Bates, Mächler, Bolker, & Walker, 2015).

In this analysis, a two-folded analytical approach was implemented. First, only the variables associated to each specific working hypothesis were tested, and the models corresponding to all combinations of those variables were built. Then, an Information Criteria approach (Akaike Information Criteria -AICc - corrected for small samples) was used, in other to perform model

selection for each hypothesis. All produced models for each hypothesis were ranked according with their ΔAICc (corresponding to the difference between each model AICc and the smaller AICc value), with models presenting ΔAICc<2 being retained as best models. The best model(s) for each hypothesis were compared using the AIC approach to test which hypothesis was more supported by the data (i.e. the model with the lowest AICc).

In the second step, we selected the variables included in the best model for each hypotheses that showed a 95% confidence interval that did not cross zero (i.e. informative variables for which is possible to assess the effect on the dependent variable – positive vs negative; (Arnold, 2010)). Those variables were used to test a combined hypothesis, which defended that antimicrobial resistance variation is driven by multiple origin factors. As more than one model fulfilled the best model criterion (i.e., ΔAICc<2), a model averaging procedure was implemented to estimate variables coefficients. Model selection procedures were implemented in R using "MuMIn" package (Barton, 2015). Best model validation was tested by the Receiver Operating Characteristic (ROC) Curve and the Area Under the Curve (AUC), using the R package "pROC" (Robin et al., 2011).

RESULTS

Staphylococcus aureus carrier status among wild ungulates

The selective culture of nasal swabs (*n*=254) from hunted wild boar (*n*=177), red deer (*n*=54) and fallow deer (*n*=23), followed by molecular identification of presumptive *S. aureus,* yielded 300 methicillin-susceptible *S. aureus* isolates, along with three MRSA isolates. The overall proportion of *S. aureus* carriers (i.e. confirmed *S. aureus* isolation in each host) was 35.8% (95% CI: 29.89-41.76%). The overall proportion of carriers by host was established at 53.7% (95% CI: 40-67.4%) for red deer and 32.2% (95% CI: 25.3-39.2%) for wild boar (Table 2). As for fallow

deer, samples were collected in one site only, in the Southern region, with an overall proportion of carriers of 21.7% (95% CI: 3.5-40%) (Table 2).

Different regions (NUTS II) of Portugal presented differences in the overall proportion of carriers. The Central region was identified as hosting significantly more *S. aureus* carriers than the North and Alentejo (Supplementary Figure 1). The Central region (Mean: 58.14%; 95% CI: 47.5-68.78%) was identified as hosting significantly (Mann-Whitney test, α=0.05; p<0.001) more *S. aureus* carriers than the North (Mean: 14.29%, 95% CI: 0-30.6%) and Alentejo (Mean 26.09%; 95% CI: 18.67-33.51%). For red deer, there was no statistical differences (Mann-Whitney test, α=0.05; p-value = 0.1542) when comparing the proportions between male and female hosts (male: 61.1% [95% CI: 44.4-77.8%]; female: 38.9% [95% CI: 13.9-63.8%]), with a p-value of 0.1542 (Mann-Whitney test), and also when comparing juveniles and adults (juveniles: 70% [95% CI: 35.4-100%]; adults: 50% [95% CI:34.6-65.4%]), with a p-value of 0.3095 (Mann-Whitney test, α=0.05). Similarly, wild boar did not show any statistical differences regarding the proportion of carriers among male and female hosts (male: 32.4% [95% CI: 21.5-43.3%]; female: 32% [95% CI: 22.8-41.2%]) (Mann-Whitney test, α=0.05; p-value=1). Only the carrier proportion of wild boar juveniles and adults were significantly different (juveniles: 22.4% [95% CI: 12.1-32.6%]; adults: 38.2% [95% CI:28.9-47.4%]) (Mann-Whitney test, α=0.05; p-value=0.0320).

Molecular diversity of *Staphylococcus aureus* from wild ungulates

The *spa* typing analysis was successfully applied to 277 out of *S. aureus* 303 isolates. We repeated PCR analyses for the non-typeable 26 isolates testing different sets of reactional and thermal profiling conditions, but *spa* amplification for those isolates was unfortunately not achieved, even though molecular identification as *S. aureus* was confirmed by PCR. The 277 typeable isolates yielded 26 *spa* types (Table 2), the most predominant (top five) being t11502 (n=82 isolates), t12939 (n=26), t3750 (n=22), t7386 (n=18) and t002 (n=17) (Figure 1). The

296 presence of nine isolates from t011 and eight isolates from t034, usually associated to
297 livestock, was also registered in wild boar and fallow deer. The analysis of the distribution of
298 *spa* types per host species evidenced that both wild boar and red deer share t11502 as the
299 most frequent *spa* type. From the top five, wild boar isolates represent all the isolates from
300 t12939 (*n*=26) and also account for the majority of isolates from *spa* types t11502 (*n*=37), t3750
301 (*n*=18), t7386 (*n*=15), and t002 (*n*=15). As for red deer, all isolates from t6386 (*n*=16) were
302 retrieved from this host species, while fallow deer isolates were characterized by three *spa*
303 types only, namely t127 (*n*=6), t3583 (*n*=4) and t002 (*n*=2). Since up to 8 colonies with
304 distinctive morphology were peaked from each swab enrichment in mannitol salt agar for
305 further characterization, we registered that among the 254 animals surveyed, only twelve
306 harboured more than one *spa* type *S. aureus*. These six wild boar and six red deer correspond
307 to 11% and 21% of overall wild boar and red deer *S. aureus* carriers, respectively.

308 An exploratory approach to analyse the association of *spa* types with the available variables
309 (see methods) showed that the first two principal components (PCs) accounted for 52.9% of
310 the variance (Supplementary Table 2). Host species and sex variables were retained by the first
311 two components, with similar eigenvalues, i.e., with the variance being similarly explained
312 within both components. The variables that were more related to PC1 were thus sex, host
313 species (wild boar and red deer) and the sampling district (Leiria) (Supplementary Figure 3). As
314 for PC2, the most related variables were sex, age class, host (wild boar and red deer) and
315 sampling district (Beja) (Supplementary Figure 3). Members of *spa* type t7386 cluster together
316 in the positive axis of PC2, being more related to the variables male, adult, wild boar, and Beja
317 sampling district (Supplementary Figure 3). All those relations were found to be significant
318 (Supplementary Table 3) except for wild boar (*p*-value = 0.1832). This host species does not
319 seem to carry significantly more isolates belonging to t7386 than the remaining. Similarly,
320 members of *spa* type t002 and t12939 cluster together on the positive axis of PC1, being more
321 related to the variables female, wild boar, and Portalegre and Guarda sampling districts

(Supplementary Figure 3). While for *spa* type t002, none of the variables indicated by the exploratory analysis revealed significant differences (i.e., p>0.05). For *spa* type t12939, all variables, with the exception of sex, showed a significant influence, and a negative relation of Guarda district with this *spa* type was highlighted (*p*-value = 0.0156) (Supplementary Table 3). The *spa* type t11502 was more frequent in the Central region, even though its presence was noted in almost all sampled districts (Figure 2). Similarly, *spa* type t6386, which was exclusively found in red deer, is more frequent in the Central region as well, since this host is more represented in this region (Figure 2).

Since *spa* type/sequence type pairs are very frequently linked, with most *spa* types being associated to a particular sequence type, MLST analysis was applied to 20 isolates (nine from wild boar, eight from red deer, and three from fallow deer) representing the diversity of the most common *spa* types (i.e. over 1% frequency). These 19 *spa* types were grouped into ten different STs. The presumptive ST of the remaining isolates was inferred from the ST of the representative isolate within each *spa* type, similarly to what has been previously adopted in several works (Porrero, Mentaberre, et al., 2014; Ruiz-Ripa et al., 2019). Thus, the most common STs were presumably ST2678 (*n*=112), ST425 (*n*=31), ST1 (*n*=25), ST2328 (*n*=24), ST544 (*n*=18) and ST398 (*n*=17) (Table 2). Of the ten STs, only ST772 was found exclusively in wild boar, with the remaining ST being shared by two or three host species. Fallow deer was found to carry members of ST1, ST133 and ST5. Red deer hosted members of ST2678, ST425, ST1, ST2328, ST133, ST398, ST72, and ST544. Wild boar hosted isolates from each sequence type found. The ten STs detected cluster into five CCs, of which three are strongly associated to human infection (CC1, CC5 and CC8), while two are related with infection in livestock (CC133 and CC398). According to the multivariate analysis performed to examine the association of STs with the available variables, the first two PCs accounted for 51.4% of the variance (Supplementary Table 2). The variables that were more related to PC1 were sex, host (wild boar and red deer) and the sampling district (Leiria) (Supplementary Figure 4). As for PC2, the

more related variables were sex, age class and host (wild boar and red deer) (Supplementary Figure 4). When clustered according to STs in the PCA, isolates from ST544 were the only ones presenting a restricted association with variables male, adult, and wild boar, while the remaining top 6 STs presented a wider distribution in the first two PCs (Supplementary Figure 4). Both adult trait and wild boar as host species were correlated with ST544 (Supplementary Table 3). Sequence types do not appear to present any geographical bias (Supplementary Figure 5).

The overall discriminatory power of both techniques was high, with *spa* typing (D=0.8774) presenting higher power than MLST (D=0.7879). Although the number of isolates subjected to *spa* typing was higher than those effectively submitted to MLST, it should be reminded that the discriminatory power accounts for the number of types and their relative abundance. Concerning allelic diversity of each MLST *locus,* the values ranged from 0.6903 to 0.7865, with *yqiL* being the most discriminatory: *pta* (h=0.6903), *glpF* (h=0.7098), *tpi* (h=0.7273), *arcC* (h=0.7355), *gmk* (h=0.7562), *aroE* (h=0.7614) and *yqiL* (h=0.7865).

Antimicrobial resistance phenotypes and genotypic resistance markers

Concerning antimicrobial resistance, the phenotypic characterization of 303 isolates led to the identification of 91 *S. aureus* resistant to at least one antimicrobial agent according to EUCAST clinical criteria. From those, ten were classified as multidrug resistant, three being positive for the detection of *mecA* resistance marker and the remaining presenting a non-wild type phenotype to at least one antimicrobial agent from at least three different categories (Table 3). The *mecC* marker was not detected among the 303 *S. aureus* isolates. All multidrug resistant isolates, with the exception of one isolate from red deer, were retrieved from wild boar (Table 2). Additionally, one isolate exhibited a MIC of 4 mg/L for vancomycin, which although being considered wild-type (i.e. susceptible) according to EUCAST clinical criteria (EUCAST, 2021), stands right at the MIC breakpoint value above which resistance (non-wild type) is considered

(Table 3). The antimicrobials to which phenotypic resistance was more frequent were penicillin (27.1% of isolates), followed by erythromycin (6.9%), tetracycline and cefoxitin (4.3%), gentamicin and oxacillin (3.3%), ciprofloxacin (1.7%) and linezolid (1.0%) (Supplementary Table 1). In the whole set of 303 isolates, resistance to either trimethoprim-sulfamethoxazole or vancomycin was not detected. Isolates from different hosts presented different patterns of resistance: for cervids, resistance to benzylpenicillin (25.9%), tetracycline (7.4%), oxacillin (3.7%), cefoxitin (1.9%) and ciprofloxacin (0.9%) was registered. The isolates from wild boar presented resistance to all antimicrobials from the selected panel, with the exception of trimethoprim-sulfamethoxazole and vancomycin, as referred above. Principal component analysis of resistance phenotypes categories was completed: isolates were classified into three categories according to their antimicrobial resistance phenotype: "Susceptible" for fully susceptible isolates, "Resistant" for isolates resistant to at least one antimicrobial agent and "Multidrug resistant" for isolates defined as such, according to the definition previously defined. The first two PCs account for 50.9% of the variance (Supplementary Table 2), showing that the variables more related to PC1 were sex, host (wild boar and red deer) and the sampling district (Leiria) (Supplementary Figure 6). As for PC2, the most related variables were sex, age class and sampling district (Portalegre and Leiria) (Supplementary Figure 6). When clustered, multidrug resistant (MDR) isolates exhibited a restricted association to variables adult, female and wild boar (Supplementary Figure 6), with a strong correlation of MDR to the female trait supported by a statistical test (p-value = 0.0005) (Supplementary Table 3). The highest number of antimicrobial resistant isolates were retrieved from hosts in sampling site number 33, located in Beja district (n=29), sampling site number 21, located in Setubal district (n=13) and sampling site number 19, in Portalegre district (n=14).

Results from molecular typing and antimicrobial resistance are summarized in Figure 3.

Ecological modelling to unravel significant predictors of AMR occurrence

The data presented spatial autocorrelation (Moran's I = 0.057; *p*-value < 0.001) indicating the need to use the variable Municipality as a random effect in the GLMM, to account for this data structure. Multicollinearity was confirmed for variables Forest_cov, Cattle_abund and Driest_prec, which were then discarded from the corresponding hypothesis due to the high VIF values (VIF>5). Thus, the combinations of the following variables were tested for each hypothesis: H1) Land Use, H2) Livestock abundance, H3) Anthropogenic disturbance, H4) Wild ungulates presence, H5) Life-history traits and H6) Climate. The hypothesis with the highest support was the combined hypothesis, which registered lowest overall AICc (Table 4). However, four models within this hypothesis showed a ΔAICc<2, and therefore we estimated an average model representing the most parsimonious and explanatory hypothesis to describe the presence/absence of antimicrobial resistance in *S. aureus* isolates. Those four models were composed by a combination of four variables: Agriculture, Sex, Swine and Mouflon. The average model highlights the influence of Agricultural land cover, swine abundance and sex as the predictors of antimicrobial resistance, with 95% confidence intervals that did not cross zero. Thus, areas with higher proportion of agricultural lands and swine abundance have a higher probability of antimicrobial resistance occurrence (Supplementary Table 4). Furthermore, females were also more prone to host antimicrobial resistance strains than males, supporting the preliminary predictions by the use of PCA (Supplementary Table 4). The AUC value derived from the ROC curve reached 0.8601, revealing good accuracy of the average model to predict the occurrence of antimicrobial resistance in *S. aureus* isolates (Figure 4) (Manel, Williams, & Ormerod, 2001).

DISCUSSION

The global dissemination of ARG and AMR bacteria presents a global threat to public and ecosystem health. Most studies on *S. aureus* have focused the human and veterinary settings, while the environmental sphere including wildlife, has been neglected. However, to

understand the circulation of antimicrobial resistance determinants, including those specifically associated to *S. aureus* ecology, it is the key to expand surveillance at the interface of natural and humanized environments. Wild ungulates offer that opportunity, which was thus explored in this study. They may establish the link between different environments (e.g. urban, natural and semi-natural) through a complex web of interactions, as they have large home ranges, are untreated with antibiotics, and their habitat can overlap with livestock and humans (Torres, Fernandes, et al., 2020). Furthermore, distinctive ecological features (e.g. feeding habits, space use) and life history traits, like the foraging and opportunistic feeding behaviour of wild boar near human settings, which contrasts with human avoidance among deer, makes them good models to assess anthropogenic influence on AMR dissemination. Our results show that wild ungulates carry *S. aureus* at a relatively high proportion. These results are in line with others across Europe (Monecke et al., 2016; Porrero, Mentaberre, et al., 2014; Ruiz-Ripa et al., 2019; Seinige et al., 2017). They show different carrier proportions among the different surveyed host species, but also highlight geographical differences. In this work, there is a higher representability of the Central to South region of Portugal, less explored so far in the few existing studies. Differences in the overall proportion of carriers between regions was verified. Interestingly, red deer is also more abundant at the Centre. The estimated carriage percentages point to a proportion of 32.2% carriers amongst wild boar in mainland Portugal. Likewise, in Spain, the percentage of carriers has been reported to range from 17.7% to 65.0%, depending on the surveyed geographical area, with the first work including ten different Spanish provinces, while the later was focused on one region only (Porrero, Mentaberre, et al., 2014; Ruiz-Ripa et al., 2019). Other European countries like Germany, Austria or Sweden have also reported wild boar as a carrier of *S. aureus* (Monecke et al., 2013; Seinige et al., 2017), highlighting the role of this species as a maintenance host. As for red deer, we found that 53.7% were carriers of *S. aureus*, which is higher than the average rates reported in Spain for red deer under semi-extensive farming, ranging from 24.6% to 44% (Gómez et al., 2015; Ruiz-Ripa et al.,

2019). As for wild red deer, the reported values in other countries range from 19.78% in Spain to 90.67% in Italy (Luzzago et al., 2019; Porrero, Mentaberre, et al., 2014). The latter prevalence rate represents an extreme scenario, in which an active culling program was applied thereafter due to overabundance of red deer in a specific region. Both wild boar and red deer have been reported to be increasing their population density across Europe, with the Iberian Peninsula reflecting such trend (Massei et al., 2015). The higher population density and changes in landscape cover have been possibly promoting the contact of wild ungulates with *S. aureus*, namely through the presence of wild boar, for which the foraging and feeding behaviour may promote a closer contact with human populations. Altogether, these studies suggest that *S. aureus* carriage in wild red deer in Portugal may be experiencing an increasing trend as well, along with host population density. Red deer are gregarious species, living in family groups, particularly the females, which could thus have spill over effects in other sympatric species (Palomo, Gisbert, & Blanco, 2007). Regular monitoring should thus be assured in the future.

Concerning the molecular characterization of the 303 *S. aureus* isolates, the most frequent *spa* types were t11502 (*n*=82), t12939 (*n*=26), t3750 (*n*=22), t7386 (*n*=18) and t002 (*n*=17). We also found one of the most frequent *spa* types across Europe, *spa* type t3750. This molecular type has been reported as the most frequent in wild boars in Spain (Porrero, Mentaberre, et al., 2014) and, more recently, in Portugal (Sousa et al., 2017). Correspondently, t11502/ST2678 and t12939/ST2678 presumably represent around one third of the total set of isolates retrieved, being previously reported only in Spain (Heaton et al., 2020; Porrero, Mentaberre, et al., 2014). Also strongly represented was ST425, more commonly found in animals, ranging from small mammals, like rabbits, *Oryctolagus cuniculus* (Vancraeynest et al., 2006), or badgers, *Meles meles* (Monecke et al., 2016), to wild boars (Porrero, Mentaberre, et al., 2014; Porrero, Valverde, et al., 2014; Seinige et al., 2017), red deer (Monecke et al., 2016), roe deer, *Capreolus capreolus* (Porrero, Valverde, et al., 2014), or Iberian Ibex, *Capra pyrenaica* (Porrero,

476 Mentaberre, et al., 2014). In contrast, we also detected the presence of STs clustered in CCs
477 like CC1, CC5 or CC8, which are more commonly associated to humans. However, those STs
478 have also been found associated with particular animal hosts, like CC5 with poultry (Nübel et
479 al., 2008) or CC8 with bovines (Resch et al., 2013), via host species jump events. In Portugal,
480 the genetic diversity of *S. aureus* isolates from different hosts was evaluated in a study that
481 reported humans hosting *S. aureus* belonging to CC22, CC5, CC15, CC8, CC398, CC30 and CC45
482 (Salgueiro et al., 2020) strains. As for the animal component, CC5, CC1, CC398, CC30, ST130
483 and ST121 strains were isolated (Salgueiro et al., 2020). We also found some of those molecular
484 types in this work, namely CC1, CC5, CC8 and CC398. It should be noted that in the previous
485 study, CC8 was exclusively linked to humans, while in our work we identified CC8 in wild
486 ungulates as well. Such molecular diversity in wildlife raises questions on the origin of those
487 strains, and on how sampled hosts may have interacted with livestock or humans.

488 Three MRSA isolates encoding *mecA* and belonging to *spa* type t7386/ST544 were detected.
489 The set of 303 isolates was also tested for the presence of *mecC* resistance determinant, but
490 none of the retrieved isolates presented amplification of this gene. Nevertheless, this
491 determinant was previously linked to CC130 and ST425 (García-Álvarez et al., 2011), with the
492 latter being one of the most frequent molecular types in this study. This finding may also be
493 linked to the fact that *mecC* appears to have a low prevalence, even amongst animals, as
494 estimated in a meta-analysis from 2016 that establishes *mecC* prevalence at 0.10% (Diaz,
495 Ramalheira, Afreixo, & Gago, 2016). Additionally, we found discordant results concerning the
496 lack of *mec* amplification in cefoxitin resistant isolates by agar disk diffusion, for which we were
497 not able to clarify the source. To further explore the genetic region around *mecA*, whole
498 genome sequencing will be applied to these isolates in the near future. The results of
499 antimicrobial susceptibility testing to selected antimicrobials revealed that around one third
500 of retrieved isolates were non-wild-type. Benzylpenicillin was the drug to which most isolates
501 were resistant, with almost one third of 303 isolates exhibiting a non-wild-type phenotype, in

agreement with previous studies from Europe and northern Africa (Gómez et al., 2015; Mairi et al., 2019; Porrero, Mentaberre, et al., 2014; Sousa et al., 2017). Three phenotypically linezolid-resistant isolates were detected in wild boar and red deer. In agreement, linezolid-resistant *S. aureus* was recently reported in the North of Portugal and amongst healthy pigs (Leão, Amaro, Albuquerque, & Clemente, 2021; Sousa et al., 2017). The increasing description of linezolid-resistant *S. aureus* amongst wild and farmed animals has suggested that the use of antimicrobials in the treatment of human infections, leads to consequences that exceed widely the healthcare setting, as it is the case of MRSA (Kang et al., 2020; Sousa et al., 2017). This trend in the animal counterpart has followed the increasing pattern of usage of linezolid in the treatment of human MRSA infections in recent years (Matrat et al., 2020).

Although one third of *S. aureus* herein retrieved were resistant to one antimicrobial and some showed phenotypic resistance to linezolid and/or cefoxitin, very few were MDR, suggesting that most of these antibiotic resistance phenotypes may revert in the absence of antibiotic pressure. To mitigate these selective pressures, xenobiotic containment buffers across the environment are crucial. Most antimicrobial resistant isolates presumably belong to ST544 (*n*=18), ST2678 (*n*=18), ST5 (*n*=14), based on the inference of presumptive ST from the corresponding *spa* type representative. Those STs have been generally associated with human settings, like community or healthcare (Lin et al., 2017; Ochoa et al., 2020; Ruffing et al., 2017), highlighting the potential acquisition of antimicrobial resistance determinants by direct or indirect (Matuszewska et al., 2020) contact with humans. This contact may indeed be unexpectedly relevant since ST544, which harbours the higher number of the antimicrobial resistant isolates in our study, had never been previously reported in livestock or wildlife to the extent of our knowledge.

Potential interactions between wildlife and livestock may explain the presence of several molecular types associated to livestock amongst the wildlife animals sampled in this study. In

Portugal, wild boar and red deer are the main big game species, with free-ranging populations, but also may be maintained in high densities in fenced estates, coexisting with cattle farming (Gonçalves, Alcobia, Simões, & Santos-Reis, 2012). With generalist omnivorous behaviour, wild boar opportunistically feed on crops on agricultural lands, providing opportunities for indirect exposure to humans and human activities, and indirectly contact with livestock at aggregation spots for feeding and watering (Johann, 2020; Miller, 2017). Similarly, red deer seems to adapt its behaviour and habitat use when in direct contact with humans (Coppes, 2017). Many recreational areas, such as hunting grounds, and human forested areas overlap geographically with red deer's habitat, promoting temporary avoidance of areas with frequent human presence (Coppes, 2017). Nonetheless, red deer is suggested to develop habituation to human presence, mainly in regions where they are not hunted (Coppes, 2017). Most interactions between livestock and wild ungulates are found to be indirect through the partitioned use of resources (Carrasco-Garcia et al., 2016).

Artificial feed and water supplies devised for livestock in pastures and within farms can be particularly attractive for wildlife, fostering direct and indirect species interactions which is the case of Alentejo region (Carrasco-Garcia et al., 2016). Supplementary feeding and baiting in hunting estates are also common practices, increasing anthropogenic influence exerted upon wildlife (Laguna et al., 2021). Simultaneously, in North and Central Portugal, extensively managed livestock frequently share aggregation points, namely feed and water supplies, with wild ungulates, promoting indirect contacts and opportunities for spill over. In agreement with these notions, the percentage of isolates retrieved from wildlife that share molecular types more commonly associated to livestock ascends to 23.5% in this work, while 19.13% share human related molecular types. In parallel, the fact that most AMR isolates, as well as the *mecA*-encoding MRSA, belong to human-associated molecular types provide indication of human disturbance exerted upon wildlife, namely through additive feeds containing xenobiotics for metaphylactic purposes in livestock farms (e.g., aminoglycosides, polymyxins,

553 penicillin and tetracyclines) and also through the environmental contamination of soils and
554 watersheds. In fact, reinforcing the relevance of human disturbance upon ecological settings,
555 our modelling approaches clearly indicate that agricultural land cover and livestock production
556 (i.e. swine abundance) are linked to the occurrence of antimicrobial resistant *S. aureus*. The
557 AMR problem associated to extended use in animal husbandry is exacerbated, by poor
558 sanitation in rural settings and wastewater infrastructures that are inappropriate to deal with
559 antibiotic residues or resistant bacteria, contributing to significant environmental
560 contamination (Fouz et al., 2020). Manure application in agricultural soils leads to the
561 infiltration of xenobiotic and AMR bacteria into soil, then into groundwaters, while the direct
562 discharges of effluents into watersheds contaminate superficial waters (Silva et al., 2020).
563 These water bodies are subsequently used by wild animals and are exploited for raw water
564 uptake for human drinking purposes, potentially perpetuating the cycle of AMR
565 bacteria/xenobiotics in several spheres. Nevertheless, in our study, the presence of
566 antimicrobial resistant *S. aureus* was not related to the percentage of water bodies coverage
567 of the sampled municipalities, suggesting that other sources might be more influential in the
568 landscape context of the monitored areas in Portugal. Moreover, antimicrobials naturally
569 produced by environmental bacteria may also exert selective pressure.
570
571 **CONCLUSION**
572
573 Our results suggest that carriage of antimicrobial resistant *S. aureus* by wild ungulates may be
574 due to specific socio-ecological conditions linked to human-associated disturbance, like
575 proximity to livestock (namely, swine abundance) and landscape conversion towards
576 agricultural lands, a subject that deserves further evaluation in the future.

577 The results provided by this study highlight the relevance of maintaining a comprehensive
578 approach under the realm of One Health by dissecting the several components at the human,

579 environment and livestock interfaces that may act upon the lifecycle of antimicrobials and
580 AMR bacteria. It has become clear that even though wildlife does not come in contact with
581 antimicrobials directly, they have a role in the maintenance and dispersal of antimicrobial
582 resistance determinants. The fact that some resistance determinants, like *mecA*, or the
583 presence of linezolid-resistant isolates, that are usually linked to human-associated strains are
584 also present in wild boar and red deer, reveals that additional measures must be taken in order
585 to prevent the indirect sources of contamination. There is an obvious need to mitigate routes
586 of transmission of AMR bacteria and ARG to wildlife, with particular priority to epidemiological
587 surveillance studies that integrate the environmental context to understand the complex
588 dynamics of AMR and then, intervene. Prevention of further spread of AMR bacteria is of the
589 utmost importance and will require a multidisciplinary approach involving all stakeholders.

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ETHICAL APPROVAL

Ethical review and approval were waived for this study since the samples analysed here were obtained in the scope of recreational hunting activities and donated for scientific purposes. No animals were sacrificed for the purpose of this study. None of the authors were responsible for the death of any animal. All applicable institutional and/or national/international guidelines for the use of animal specimens have been followed.

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REFERENCES

Arnold, T. W. (2010). Uninformative Parameters and Model Selection Using Akaike's Information Criterion. *The Journal of Wildlife Management, 74*(6), 1175-1178. doi: https://doi.org/10.1111/j.1937-2817.2010.tb01236.x

Barton, K. (2015). MuMIn: Multi-Model Inference. R package version 1.43.17.

Bates, D., Mächler, M., Bolker, B., & Walker, S. (2015). Fitting Linear Mixed-Effects Models Using lme4. *2015, 67*(1), 48 %J Journal of Statistical Software. doi: 10.18637/jss.v067.i01

Bencatel J., Sabino-Marques H., Álvares F., Moura A.E., & Barbosa A.M. (2019). *Atlas de Mamíferos de Portugal*. Universidade de Évora.

Bikandi, J., Millán, R. S., Rementeria, A., & Garaizar, J. (2004). In silico analysis of complete bacterial genomes: PCR, AFLP–PCR and endonuclease restriction. *Bioinformatics, 20*(5), 798-799. doi: 10.1093/bioinformatics/btg491 %J Bioinformatics

Carrasco-Garcia, R., Barasona, J. A., Gortazar, C., Montoro, V., Sanchez-Vizcaino, J. M., & Vicente, J. (2016). Wildlife and livestock use of extensive farm resources in South Central Spain: implications for disease transmission. *European Journal of Wildlife Research, 62*(1), 65-78. doi: 10.1007/s10344-015-0974-9

Diaz, R., Ramalheira, E., Afreixo, V., & Gago, B. (2016). Methicillin-resistant Staphylococcus aureus carrying the new mecC gene--a meta-analysis. *Diagn Microbiol Infect Dis, 84*(2), 135-140. doi: 10.1016/j.diagmicrobio.2015.10.014

Direção-Geral do Território - Ministério do Ambiente e Ação Climática - Portugal. (2018, Last Update). Carta de Uso e Ocupação do Solo para 2018.

Enright, M. C., Day, N. P., Davies, C. E., Peacock, S. J., & Spratt, B. G. (2000). Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of Staphylococcus aureus. *Journal of Clinical Microbiology, 38*(3), 1008-1015. doi: 10.1128/JCM.38.3.1008-1015.2000

EUCAST. (2021). Breakpoint tables for interpretation of MICs and zone diameters. https://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_11.0_Breakpoint_Tables.pdf

European Food Safety Authority. (2012). Technical specifications on the harmonised monitoring and reporting of antimicrobial resistance in methicillin-resistant Staphylococcus aureus in food-producing animals and food. *Efsa Journal*.

F. Dormann, C., M. McPherson, J., B. Araújo, M., Bivand, R., Bolliger, J., Carl, G., . . . Wilson, R. (2007). Methods to account for spatial autocorrelation in the analysis of species distributional data: a review. *Ecography, 30*(5), 609-628. doi: https://doi.org/10.1111/j.2007.0906-7590.05171.x

Feßler, A. T., Thomas, P., Mühldorfer, K., Grobbel, M., Brombach, J., Eichhorn, I., . . . Schwarz, S. (2018). Phenotypic and genotypic characteristics of Staphylococcus aureus isolates from zoo and wild animals. *Veterinary Microbiology, 218*, 98-103. doi: https://doi.org/10.1016/j.vetmic.2018.03.020

Fick, S. E., & Hijmans, R. J. (2017). WorldClim 2: new 1-km spatial resolution climate surfaces for global land areas. *37*(12), 4302-4315. doi: https://doi.org/10.1002/joc.5086

Fouz, N., Pangesti, K. N. A., Yasir, M., Al-Malki, A. L., Azhar, E. I., Hill-Cawthorne, G. A., & Abd El Ghany, M. (2020). The Contribution of Wastewater to the Transmission of Antimicrobial Resistance in the Environment: Implications of Mass Gathering Settings. *Tropical medicine and infectious disease, 5*(1), 33. doi: 10.3390/tropicalmed5010033

García-Álvarez, L., Holden, M. T., Lindsay, H., Webb, C. R., Brown, D. F., Curran, M. D., . . . Holmes, M. A. (2011). Meticillin-resistant Staphylococcus aureus with a novel mecA homologue in human and bovine populations in the UK and Denmark: a descriptive study. *Lancet Infect Dis, 11*(8), 595-603. doi: 10.1016/s1473-3099(11)70126-8

Gómez, P., Lozano, C., González-Barrio, D., Zarazaga, M., Ruiz-Fons, F., & Torres, C. (2015). High prevalence of methicillin-resistant Staphylococcus aureus (MRSA) carrying the mecC gene in a semi-extensive red deer (Cervus elaphus hispanicus) farm in Southern Spain. *Vet Microbiol, 177*(3-4), 326-331. doi: 10.1016/j.vetmic.2015.03.029

Gonçalves, P., Alcobia, S., Simões, L., & Santos-Reis, M. (2012). Effects of management options on mammal richness in a Mediterranean agro-silvo-pastoral system. *Agroforestry Systems, 85*(3), 383-395. doi: 10.1007/s10457-011-9439-7

Heaton, C. J., Gerbig, G. R., Sensius, L. D., Patel, V., & Smith, T. C. (2020). Staphylococcus aureus Epidemiology in Wildlife: A Systematic Review. *Antibiotics-Basel, 9*(2), 28. doi: 10.3390/antibiotics9020089

Hoekstra, J., Zomer, A. L., Rutten, V. P. M. G., Benedictus, L., Stegeman, A., Spaninks, M. P., . . . Koop, G. (2020). Genomic analysis of European bovine Staphylococcus aureus from clinical versus subclinical mastitis. *Scientific Reports, 10*(1), 18172. doi: 10.1038/s41598-020-75179-2

Hunter, P. R., & Gaston, M. A. (1988). Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. *J Clin Microbiol, 26*(11), 2465-2466. doi: 10.1128/jcm.26.11.2465-2466.1988

Instituto Nacional de Estatística. (2019, Last Update). Efetivo animal (N.º) das explorações agrícolas por Localização geográfica (NUTS - 2013) e Espécie animal.

Instituto Nacional de Estatística. (2020, Last Update). Densidade populacional (N.º/ km²) por Local de residência (NUTS - 2013).

Johny, B. (2019). Characterization of Methicillin-resistant Staphylococcus aureus Isolated from Nearby Hospitals from two Different Countries. *J Pure Appl Microbiol., 13*(3), 1683-1688.

Kang, H. Y., Moon, D. C., Mechesso, A. F., Choi, J.-H., Kim, S.-J., Song, H.-J., . . . Lim, S.-K. (2020). Emergence of cfr-Mediated Linezolid Resistance in Staphylococcus aureus Isolated from Pig Carcasses. *9*(11), 769.

Kozajda, A., Jeżak, K., & Kapsa, A. (2019). Airborne Staphylococcus aureus in different environments-a review. *Environmental science and pollution research international, 26*(34), 34741-34753. doi: 10.1007/s11356-019-06557-1

Laguna, E., Carpio, A. J., Vicente, J., Barasona, J. A., Triguero-Ocaña, R., Jiménez-Ruiz, S., . . . Acevedo, P. (2021). The spatial ecology of red deer under different land use and management scenarios: Protected areas, mixed farms and fenced hunting estates. *Science of the Total Environment, 786*, 147124. doi: https://doi.org/10.1016/j.scitotenv.2021.147124

Laxminarayan, R., Duse, A., Wattal, C., Zaidi, A. K., Wertheim, H. F., Sumpradit, N., . . . Cars, O. (2013). Antibiotic resistance-the need for global solutions. *Lancet Infect Dis, 13*(12), 1057-1098. doi: 10.1016/s1473-3099(13)70318-9

Leão, C., Amaro, A., Albuquerque, T., & Clemente, L. (2021). *Whole Genome Sequencing of Linezolid-Resistant Staphylococcus aureus ST398 From Healthy Pigs In Portugal*. Paper presented at the OneHealth EJP 2021.

Letunic, I., & Bork, P. (2021). Interactive Tree Of Life (iTOL) v5: an online tool for phylogenetic tree display and annotation. *Nucleic acids research, 49*(W1), W293-W296. doi: 10.1093/nar/gkab301 %J Nucleic Acids Research

Lin, J., Xu, P., Peng, Y., Lin, D., Ou, Q., Zhang, T., . . . Yao, Z. (2017). Prevalence and characteristics of Staphylococcus aureus and methicillin-resistant Staphylococcus aureus nasal colonization among a community-based diabetes population in Foshan, China. *Journal of diabetes investigation, 8*(3), 383-391. doi: 10.1111/jdi.12591

Luzzago, C., Lauzi, S., Ehricht, R., Monecke, S., Trogu, T., Pedrotti, L., . . . Piccinini, R. (2019). Staphylococcus aureus nasal and intestinal carriage by free-ranging red deer: evidence of human, domestic and wild animal lineages. *International Journal of Infectious Diseases, 79*, 21-22. doi: 10.1016/j.ijid.2018.11.067

718 Magiorakos, A. P., Srinivasan, A., Carey, R. B., Carmeli, Y., Falagas, M. E., Giske, C. G., . . .
719 Monnet, D. L. (2012). Multidrug-resistant, extensively drug-resistant and pandrug-
720 resistant bacteria: an international expert proposal for interim standard definitions for
721 acquired resistance. *Clinical Microbiology and Infection, 18*(3), 268-281. doi:
722 10.1111/j.1469-0691.2011.03570.x
723 Mairi, A., Touati, A., Pantel, A., Zenati, K., Martinez, A. Y., Dunyach-Remy, C., . . . Lavigne, J.-P.
724 (2019). Distribution of Toxinogenic Methicillin-Resistant and Methicillin-Susceptible
725 Staphylococcus aureus from Different Ecological Niches in Algeria. *11*(9), 500.
726 Manel, S., Williams, H. C., & Ormerod, S. J. (2001). Evaluating presence–absence models in
727 ecology: the need to account for prevalence. *Journal of Applied Ecology, 38*(5), 921-
728 931. doi: https://doi.org/10.1046/j.1365-2664.2001.00647.x
729 Marchesi, J. R., Sato, T., Weightman, A. J., Martin, T. A., Fry, J. C., Hiom, S. J., & Wade, W. G.
730 (1998). Design and Evaluation of Useful Bacterium-Specific PCR Primers That Amplify
731 Genes Coding for Bacterial 16S rRNA. *64*(2), 795-799. doi: doi:10.1128/AEM.64.2.795-
732 799.1998
733 Marshall, B. M., & Levy, S. B. (2011). Food animals and antimicrobials: impacts on human
734 health. *Clin Microbiol Rev, 24*(4), 718-733. doi: 10.1128/cmr.00002-11
735 Martineau, F., Picard, F. J., Roy, P. H., Ouellette, M., & Bergeron, M. G. (1998). Species-specific
736 and ubiquitous-DNA-based assays for rapid identification of Staphylococcus aureus. *J*
737 *Clin Microbiol, 36*(3), 618-623. doi: 10.1128/jcm.36.3.618-623.1998
738 Massei, G., Kindberg, J., Licoppe, A., Gačić, D., Šprem, N., Kamler, J., . . . Náhlik, A. (2015). Wild
739 boar populations up, numbers of hunters down? A review of trends and implications
740 for Europe. *Pest Manag Sci, 71*(4), 492-500. doi: 10.1002/ps.3965
741 Matrat, L., Plaisant, F., Barreto, C., Claris, O., & Butin, M. (2020). Increasing use of linezolid in
742 a tertiary NICU during a 10-year period: reasons and concerns for the future.
743 *Antimicrobial Resistance & Infection Control, 9*(1), 156. doi: 10.1186/s13756-020-
744 00818-2
745 Matuszewska, M., Murray, G. G. R., Harrison, E. M., Holmes, M. A., & Weinert, L. A. (2020). The
746 Evolutionary Genomics of Host Specificity in Staphylococcus aureus. *Trends in*
747 *Microbiology, 28*(6), 465-477. doi: 10.1016/j.tim.2019.12.007
748 Monecke, S., Gavier-Widén, D., Hotzel, H., Peters, M., Guenther, S., Lazaris, A., . . . Ehricht, R.
749 (2016). Diversity of Staphylococcus aureus Isolates in European Wildlife. *PLOS ONE,*
750 *11*(12), e0168433. doi: 10.1371/journal.pone.0168433
751 Monecke, S., Gavier-Widen, D., Mattsson, R., Rangstrup-Christensen, L., Lazaris, A., Coleman,
752 D. C., . . . Ehricht, R. (2013). Detection of mecC-Positive Staphylococcus aureus (CC130-
753 MRSA-XI) in Diseased European Hedgehogs (Erinaceus europaeus) in Sweden. *PLOS*
754 *ONE, 8*(6), e66166. doi: 10.1371/journal.pone.0066166
755 Nazareth, R., Gonçalves-Pereira, J., Tavares, A., Miragaia, M., de Lencastre, H., Silvestre, J., . . .
756 Póvoa, P. (2012). Infeção por staphylococcus aureus meticilina-resistente da
757 comunidade em Portugal. *Revista Portuguesa de Pneumologia, 18*(1), 34-38. doi:
758 https://doi.org/10.1016/j.rppneu.2011.05.007
759 Nübel, U., Roumagnac, P., Feldkamp, M., Song, J.-H., Ko, K. S., Huang, Y.-C., . . . Achtman, M.
760 (2008). Frequent emergence and limited geographic dispersal of methicillin-resistant
761 Staphylococcus aureus. *Proceedings of the National Academy of Sciences, 105*(37),
762 14130. doi: 10.1073/pnas.0804178105
763 Ochoa, S. A., Cruz-Córdova, A., Mancilla-Rojano, J., Escalona-Venegas, G., Esteban-Kenel, V.,
764 Franco-Hernández, I., . . . Xicohtencatl-Cortes, J. (2020). Control of Methicillin-
765 Resistant Staphylococcus aureus Strains Associated With a Hospital Outbreak Involving
766 Contamination From Anesthesia Equipment Using UV-C. *Frontiers in Microbiology, 11*,
767 600093-600093. doi: 10.3389/fmicb.2020.600093
768 Palmeira, J. D., Cunha, M. V., Carvalho, J., Ferreira, H., Fonseca, C., & Torres, R. T. (2021).
769 Emergence and Spread of Cephalosporinases in Wildlife: A Review. *11*(6), 1765.

770 Palomo, L., Gisbert, J., & Blanco, J. (2007). Atlas Y Libro Rojo De Los Mamíferos Terrestres De
771 España.
772 Paradis, E., & Schliep, K. (2018). ape 5.0: an environment for modern phylogenetics and
773 evolutionary analyses in R. *Bioinformatics, 35*(3), 526-528. doi:
774 10.1093/bioinformatics/bty633 %J Bioinformatics
775 Peres, D., Pina, E., & Fonseca Cardoso, M. (2011). Methicillin-Resistant Staphylococcus Aureus
776 (MRSA) in a Portuguese hospital and its risk perception by health care professionals.
777 *Revista Portuguesa de Saúde Pública, 29*(2), 132-139. doi:
778 https://doi.org/10.1016/S0870-9025(11)70017-7
779 Plaza-Rodríguez, C., Alt, K., Grobbel, M., Hammerl, J. A., Irrgang, A., Szabo, I., . . . Tenhagen, B.-
780 A. (2021). Wildlife as Sentinels of Antimicrobial Resistance in Germany? *, 7*(1251). doi:
781 10.3389/fvets.2020.627821
782 Porrero, M. C., Mentaberre, G., Sánchez, S., Fernández-Llario, P., Casas-Díaz, E., Mateos, A., . .
783 . Domínguez, L. (2014). Carriage of Staphylococcus aureus by free-living wild animals
784 in Spain. *Appl Environ Microbiol, 80*(16), 4865-4870. doi: 10.1128/aem.00647-14
785 Porrero, M. C., Valverde, A., Fernández-Llario, P., Díez-Guerrier, A., Mateos, A., Lavín, S., . . .
786 Domínguez, L. (2014). Staphylococcus aureus carrying mecC gene in animals and urban
787 wastewater, Spain. *Emerg Infec Dis, 20*(5), 899-901. doi: 10.3201/eid2005.130426
788 Price, L. B., Stegger, M., Hasman, H., Aziz, M., Larsen, J., Andersen, P. S., . . . Aarestrup, F. M.
789 (2012). Staphylococcus aureus CC398: Host Adaptation and Emergence of Methicillin
790 Resistance in Livestock. *mBio, 3*(1), e00305-00311. doi: 10.1128/mBio.00305-11
791 Resch, G., François, P., Morisset, D., Stojanov, M., Bonetti, E. J., Schrenzel, J., . . . Moreillon, P.
792 (2013). Human-to-Bovine Jump of Staphylococcus aureus CC8 Is Associated with the
793 Loss of a β-Hemolysin Converting Prophage and the Acquisition of a New
794 Staphylococcal Cassette Chromosome. *PLOS ONE, 8*(3), e58187. doi:
795 10.1371/journal.pone.0058187
796 Richardson, E. J., Bacigalupe, R., Harrison, E. M., Weinert, L. A., Lycett, S., Vrieling, M., . . .
797 Fitzgerald, J. R. (2018). Gene exchange drives the ecological success of a multi-host
798 bacterial pathogen. *Nature Ecology & Evolution, 2*(9), 1468-1478. doi:
799 10.1038/s41559-018-0617-0
800 Robin, X., Turck, N., Hainard, A., Tiberti, N., Lisacek, F., Sanchez, J.-C., & Müller, M. (2011).
801 pROC: an open-source package for R and S+ to analyze and compare ROC curves. *BMC*
802 *Bioinformatics, 12*(1), 77. doi: 10.1186/1471-2105-12-77
803 Ruffing, U., Alabi, A., Kazimoto, T., Vubil, D. C., Akulenko, R., Abdulla, S., . . . Herrmann, M.
804 (2017). Community-Associated Staphylococcus aureus from Sub-Saharan Africa and
805 Germany: A Cross-Sectional Geographic Correlation Study. *Scientific Reports, 7*(1), 154.
806 doi: 10.1038/s41598-017-00214-8
807 Ruiz-Ripa, L., Alcalá, L., Simón, C., Gómez, P., Mama, O. M., Rezusta, A., . . . Torres, C. (2019).
808 Diversity of Staphylococcus aureus clones in wild mammals in Aragon, Spain, with
809 detection of MRSA ST130-mecC in wild rabbits. *Journal of Applied Microbiology,*
810 *127*(1), 284-291. doi: https://doi.org/10.1111/jam.14301
811 Salgueiro, V., Manageiro, V., Bandarra, N. M., Ferreira, E., Clemente, L., & Caniça, M. (2020).
812 Genetic Relatedness and Diversity of Staphylococcus aureus from Different Reservoirs:
813 Humans and Animals of Livestock, Poultry, Zoo, and Aquaculture. *8*(9), 1345.
814 Schaumburg, F., Alabi, A. S., Köck, R., Mellmann, A., Kremsner, P. G., Boesch, C., . . . Peters, G.
815 (2012). Highly divergent Staphylococcus aureus isolates from African non-human
816 primates. *Environmental Microbiology Reports, 4*(1), 141-146. doi:
817 https://doi.org/10.1111/j.1758-2229.2011.00316.x
818 Seinige, D., Von Altrock, A., & Kehrenberg, C. (2017). Genetic diversity and antibiotic
819 susceptibility of Staphylococcus aureus isolates from wild boars. *Comp Immunol*
820 *Microbiol Infect Dis, 54*, 7-12. doi: 10.1016/j.cimid.2017.07.003

821 Silva, V., Caniça, M., Capelo, J. L., Igrejas, G., & Poeta, P. (2020). Diversity and genetic lineages
822 of environmental staphylococci: a surface water overview. *Fems Microbiology Ecology,*
823 *96*(12). doi: 10.1093/femsec/fiaa191
824 Silva, V., Gabriel, S. I., Borrego, S. B., Tejedor-Junco, M. T., Manageiro, V., Ferreira, E., . . . Poeta,
825 P. (2021). Antimicrobial Resistance and Genetic Lineages of Staphylococcus aureus
826 from Wild Rodents: First Report of mecC-Positive Methicillin-Resistant S. aureus
827 (MRSA) in Portugal. *11*(6), 1537.
828 Silva, V., Oliveira, A., Manageiro, V., Caniça, M., Contente, D., Capita, R., . . . Poeta, P. (2021).
829 Clonal Diversity and Antimicrobial Resistance of Methicillin-Resistant Staphylococcus
830 pseudintermedius Isolated from Canine Pyoderma. *9*(3), 482.
831 Sousa, M., Silva, N., Manageiro, V., Ramos, S., Coelho, A., Gonçalves, D., . . . Poeta, P. (2017).
832 First report on MRSA CC398 recovered from wild boars in the north of Portugal. Are
833 we facing a problem? *Science of the Total Environment, 596-597*, 26-31. doi:
834 https://doi.org/10.1016/j.scitotenv.2017.04.054
835 Sousa, M., Silva, V., Silva, A., Silva, N., Ribeiro, J., Tejedor-Junco, M. T., . . . Poeta, P. (2020).
836 Staphylococci among Wild European Rabbits from the Azores: A Potential Zoonotic
837 Issue? *Journal of Food Protection, 83*(7), 1110-1114. doi: 10.4315/0362-028x.jfp-19-
838 423
839 Stegger, M., Andersen, P. S., Kearns, A., Pichon, B., Holmes, M. A., Edwards, G., . . . Larsen, A.
840 R. (2012). Rapid detection, differentiation and typing of methicillin-resistant
841 Staphylococcus aureus harbouring either mecA or the new mecA homologue
842 mecA(LGA251). *Clin Microbiol Infect, 18*(4), 395-400. doi: 10.1111/j.1469-
843 0691.2011.03715.x
844 Strube, M. L., Hansen, J. E., Rasmussen, S., & Pedersen, K. (2018). A detailed investigation of
845 the porcine skin and nose microbiome using universal and Staphylococcus specific
846 primers. *Scientific Reports, 8*(1), 12751. doi: 10.1038/s41598-018-30689-y
847 Torres, R. T., Carvalho, J., Cunha, M. V., & Fonseca, C. (2019). Antimicrobial Resistance and
848 Ecology: A Dialog Yet to Begin. *Ecohealth, 16*(3), 402-403. doi: 10.1007/s10393-019-
849 01438-y
850 Torres, R. T., Carvalho, J., Cunha, M. V., Serrano, E., Palmeira, J. D., & Fonseca, C. (2020).
851 Temporal and geographical research trends of antimicrobial resistance in wildlife - A
852 bibliometric analysis. *One Health, 11*, 100198. doi:
853 https://doi.org/10.1016/j.onehlt.2020.100198
854 Torres, R. T., Fernandes, J., Carvalho, J., Cunha, M. V., Caetano, T., Mendo, S., . . . Fonseca, C.
855 (2020). Wild boar as a reservoir of antimicrobial resistance. *Science of the Total*
856 *Environment, 717*, 135001. doi: https://doi.org/10.1016/j.scitotenv.2019.135001
857 Vancraeynest, D., Haesebrouck, F., Deplano, A., Denis, O., Godard, C., Wildemauwe, C., &
858 Hermans, K. (2006). International dissemination of a high virulence rabbit
859 Staphylococcus aureus clone. *J Vet Med B Infect Dis Vet Public Health, 53*(9), 418-422.
860 doi: 10.1111/j.1439-0450.2006.00977.x
861 Vittecoq, M., Godreuil, S., Prugnolle, F., Durand, P., Brazier, L., Renaud, N., . . . Renaud, F.
862 (2016). Antimicrobial resistance in wildlife. *53*(2), 519-529. doi:
863 https://doi.org/10.1111/1365-2664.12596
864 Zuur, A. F., Hilbe, J. M., & Ieno, E. N. (2013). *A beginner's guide to GLM and GLMM with R.*:
865 Newburgh: Highland Statistics Ltd.

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871 **Table 1 – Modelling hypothesis, rationale, prediction, and variable description.** All variables
872 were represented according to municipality administrative regions.

Hypothesis	Rationale	Prediction	Variables	Variables' code
H1 Land Use	The differential profile of land use along municipalities may influence the overall AMR occurrence and dispersion, as different species habit particular types of land cover	We expect higher percentage of agricultural areas and water bodies to influence positively the AMR occurrence	Agriculture coverage (%) Artificial areas coverage (%) Forest coverage (%) Water bodies coverage (%)	*Agri_cov* *Artif_cov* *Forest_cov* *Water_cov*
H2 Livestock abundance	The sympatry between livestock and wildlife animals is known to promote disease transmission	We expect higher livestock abundance to promote the AMR occurrence	Cattle abundance (no. animals) Swine (no. animals) Poultry (no. animals)	*Cattle_abun* *Swi_abun* *Poul_abun*
H3 Anthropogenic disturbance	The anthropogenic influence is known to be a factor that promotes the transmission of AMR bacteria and AMR determinants to the animal compartment	We expect that higher human density will increase AMR occurrence	Population density (no. people/km^2)	*Pop_dens*
H4 Wild ungulates presence	The interspecies transmission of AMR bacteria and AMR determinants is known to promote the maintenance within the animal compartment	We expect that the presence of different wild species will increase the AMR occurrence	Mufflon presence (binary) Roe deer presence (binary) Red deer presence (binary)	*Muff_pre* *Roe_pre* *Red_pre*
H5	The life history traits of the host,	We expect that a	Host species (Wild boar, red	*Host_spe*

Life-history traits	namely its species, sex or age can be associated with the AMR occurrence	particular species, sex, or age class is more prone to promote AMR occurrence	deer, fallow deer) Sex (Male, Female) Age (Adult, Juvenile)	 *Sex* *Age*
H6 Climate	The climate, namely the volume of precipitation, can influence animal feeding behaviour	We expect that driest zones will increase the AMR occurrence	Annual precipitation (mm) Driest month precipitation (mm) [Data were calculated by interpolations of observed data, representing average values for the period 1970–2000]	*Annual_prec* *Driest_prec*

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874 **Table 2 - *Staphylococcus aureus* detection and molecular characterization.**

Animal species	No. tested animals	No. positive animals	Prevalence	MLST[a] (No. of isolates)	MLST[b] (No. of isolates)	*spa* type (No. of isolates)
Fallow deer	23	5	21.7% (95% CI: 3.5-40%)	ST1 (1)	ST1 (6)	t127 (6)
				ST5(1)	ST5 (2)	t002 (2)
				ST133 (1)	ST133 (4)	t3583 (4)
Red deer	54	29	53.7% (95% CI: 40-67.4%)	t9632/ST1 (1) t1533/ST1 (1) t2207/ST1 (1)	ST1 (13)	t9632 (7), t1533 (5), t2207 (1)
					ST133 (3)	t3583 (3)
				ST425 (1)	ST425 (16)	t6386 (16)
					ST544 (3)	t7386 (3)
				t3750/ST2328 (1)	ST2328 (4)	t3750 (4)
				t9304/ST2678 (1) t11502/ST2678 (1)	ST2678 (49)	t11502 (45), t9304 (4)
						t1793 (1)
						t701 (2)
						t10205 (1)

Wild boar	177	57	32.2% (95% CI: 25.3-39.2%)	t1407/ST1 (1)	ST1 (6)	t1407 (4), t1533 (2)
					ST5 (15)	t002 (15)
				ST8(1)	ST72 (8)	t148 (8)
				t1181/ ST133 (1)	ST133 (4)	t3583 (3), t1181 (1)
				t011/ST398 (1)	ST398 (17)	t011 (9), t034 (8)
				t11232/ST425 (1)	ST425 (19)	t11232 (15)
				ST544 (2)	ST544 (15)	t7386 (3*, 12)
				ST772 (1)	ST772 (2)	t098 (2)
				t11230/ST2328 (1)	ST2328 (20)	t3750 (18), t11230 (2)
					ST2678 (63)	t11502 (37), t12939 (26)
						t1773 (1)
						t4279 (2)
						t11225 (2)

[a]Experimentally determined MLST (20 isolates); [b]Inferred sequence type based on presumable *Spa* type/ST associations (comprises all isolates, including [a]); * represents MRSA isolates.

Table 3 – Resistance profile of multidrug resistant *Staphylococcus aureus* isolates. GEN – Gentamicin; LIN – Linezolid; TET – Tetracycline; CXI – Cefoxitin; CIP – Ciprofloxacin; BEN – Benzylpenicillin; OXA – Oxacillin; ERY – Erythromycin.

Host	Isolate ID	Resistance phenotype	*mecA*	*mecC*
Wild boar	0645	GEN-LIN-TET	-	-
Wild boar	0649	CXI-CIP-GEN-BEN-OXA	-	-
Wild boar	0650	CXI-CIP-GEN-LIN-BEN-OXA	-	-
Wild boar	0651	CXI-CIP-GEN-BEN-OXA	-	-
Wild boar	0698	CXI-CIP-GEN-BEN	-	-
Wild boar	0959	CXI-BEN-OXA	+	-
Wild boar	0960	CXI-BEN-OXA	+	-
Wild boar	0980	CXI-ERY-GEN-BEN	-	-
Wild boar	1012	CXI-GEN-TET-BEN-OXA	-	-
Wild boar	1045	CXI-ERY-BEN	+	-

Table 4 – Best models for each of the working hypothesis selected according to the model's AICc and ΔAICc.

Models	df	AICc	ΔAICc	AICc weight
H1 – Land Use hypothesis				
Agriculture + (1|Municipality)	3	259.0	0.00	0.549
Agriculture + Water bodies + (1|Municipality)	4	260.6	1.60	0.246
Agriculture + Artificial + (1|Municipality)	4	261.0	1.98	0.205
H2 – Livestock abundance hypothesis				
Swine + (1|Municipality)	3	263.0	0.00	0.560
Swine + Poultry + (1|Municipality)	4	264.7	1.71	0.238
H3 – Anthropogenic disturbance hypothesis				
Population density + (1|Municipality)	3	264.6	0.00	1.000
H4 – Wild ungulates presence hypothesis				
Mufflon + (1|Municipality)	3	259.9	0.00	0.721
Mufflon + Roe deer + (1|Municipality)	4	261.8	1.89	0.279
H5 – Life-history traits hypothesis				
Sex + (1|Municipality)	3	262.72	0.00	0.48
Sex + Age + (1|Municipality)	4	263.84	1.13	0.27
Sex + Host + (1|Municipality)	5	264.0	1.28	0.25
H6 – Climate hypothesis				
Annual precipitation + (1|Municipality)	3	261.5	0.00	1.000
H7 – Multiple variable hypothesis				
Agriculture + Sex + Swine + (1|Municipality)	**5**	**252.9**	**0.00**	**0.345**
Sex + Swine + Mufflon + (1|Municipality)	**5**	**253.2**	**0.31**	**0.295**
Agriculture + Sex + Swine + Mufflon + (1|Municipality)	**6**	**253.8**	**0.88**	**0.222**
Agriculture + Sex + (1|Municipality)	**4**	**254.8**	**1.82**	**0.138**

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Figure Captions

Figure 1 - Minimum spanning tree of *spa* types of *Staphylococcus aureus* isolates from wildlife species. Illustration of the evolutionary link between *S. aureus* isolates based on combined character data of *spa* typing. Node size is proportional to the number of isolates belonging to each *spa* type. Node colours correspond to host species: wild boar – green; red deer – purple; and fallow deer – orange. Shading represents clonal complexes with human-associated CCs coloured in reds and livestock-associated CCs coloured in blues.

Figure 2 – *Spa* type distribution across mainland Portugal districts. Pie charts represent the proportion of each *spa* type in the pointed district. Top 15 most frequent *spa* types countrywide are represented in colours. Districts represented in the map host ten or more isolates. Map was generated with QGIS software v3.16.

Figure 3 – Molecular diversity and antibiotic resistance profiling of *Staphylococcus aureus* isolates from wildlife species.

Figure 4 – Receiver Operating Characteristic Curve (ROC) for the average model of the best hypothesis. Area Under the Curve (AUC) = 0.8601

Supplementary Tables

Supplementary Table 1 - Complete characterization of the 303 *S. aureus* isolates by Sampling site, Host, Host's sex and age class, Sequence type, *Spa* type, Resistance phenotype and *mecA* and *mecC* amplification results.

Supplementary Table 2 – Principal Components Analysis with individual and cumulative proportions of variance and explanatory variables.

Supplementary Table 3 – *p*-values of Mann-Whitney non-parametric test (α=0.05) for hypotheses testing of explanatory variables derived from Principal Components Analysis.

Supplementary Table 4 - Confidence intervals (95%) of the average model for the best hypothesis.

Figure 1

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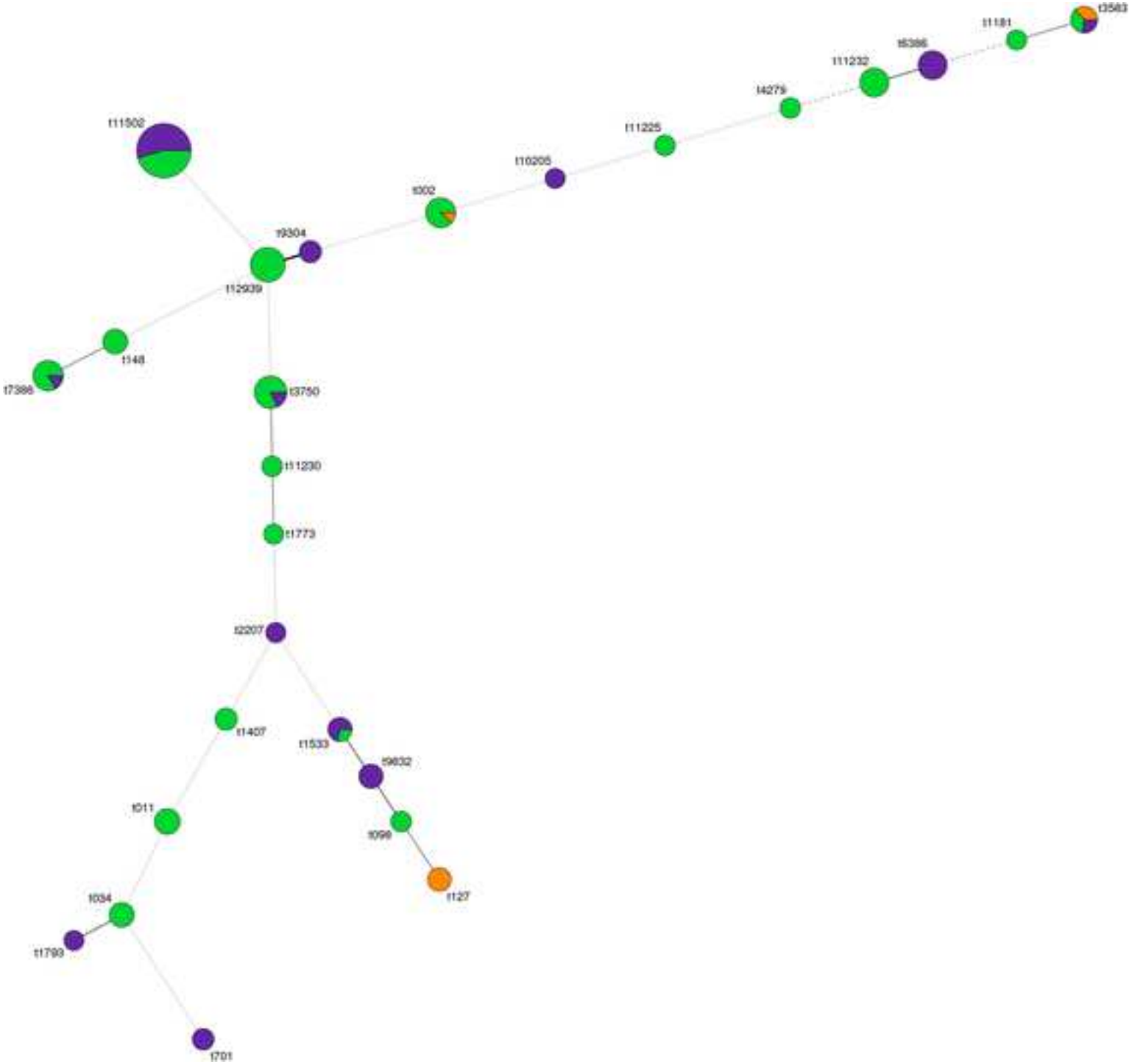

Figure 2

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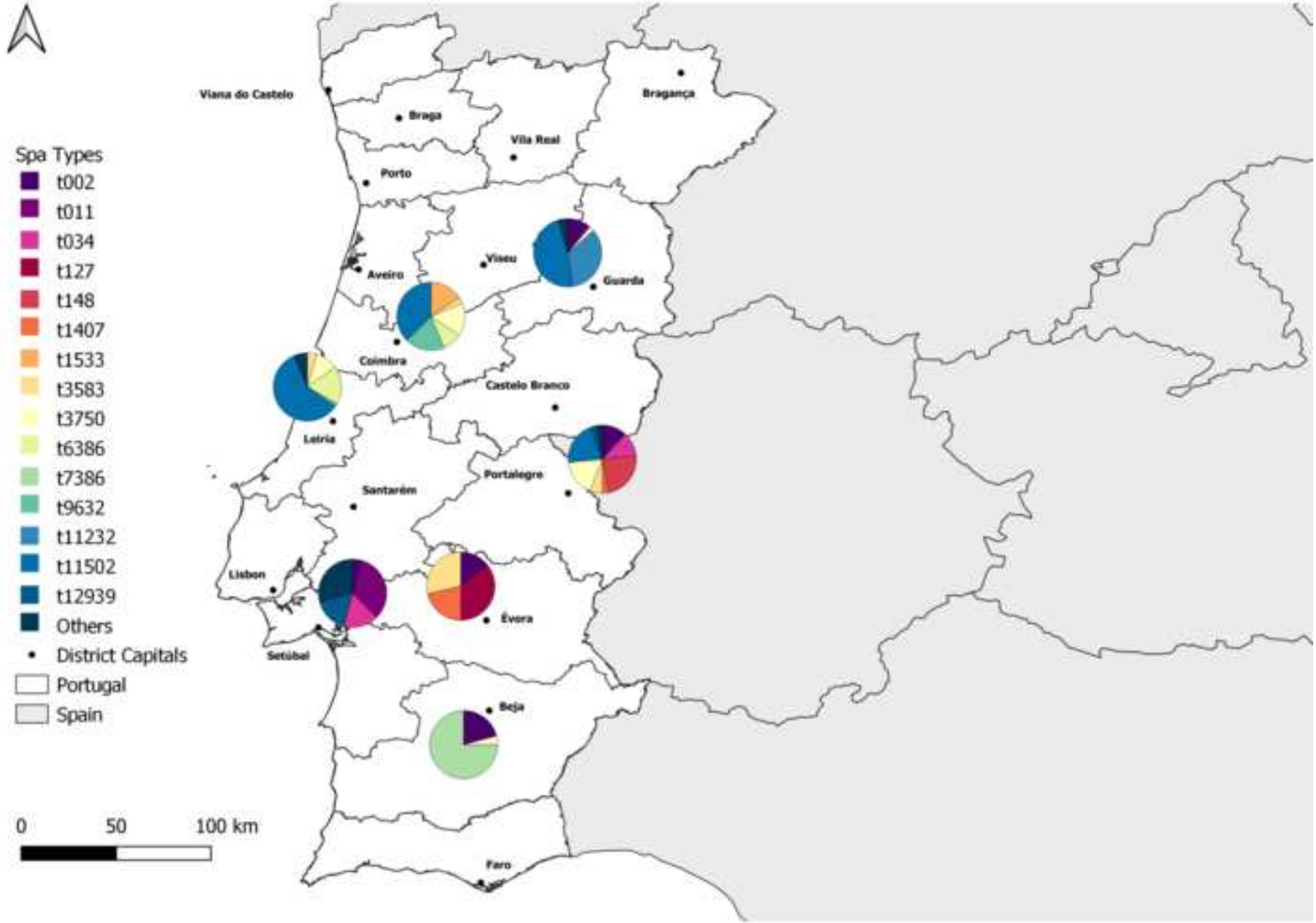

Figure 3

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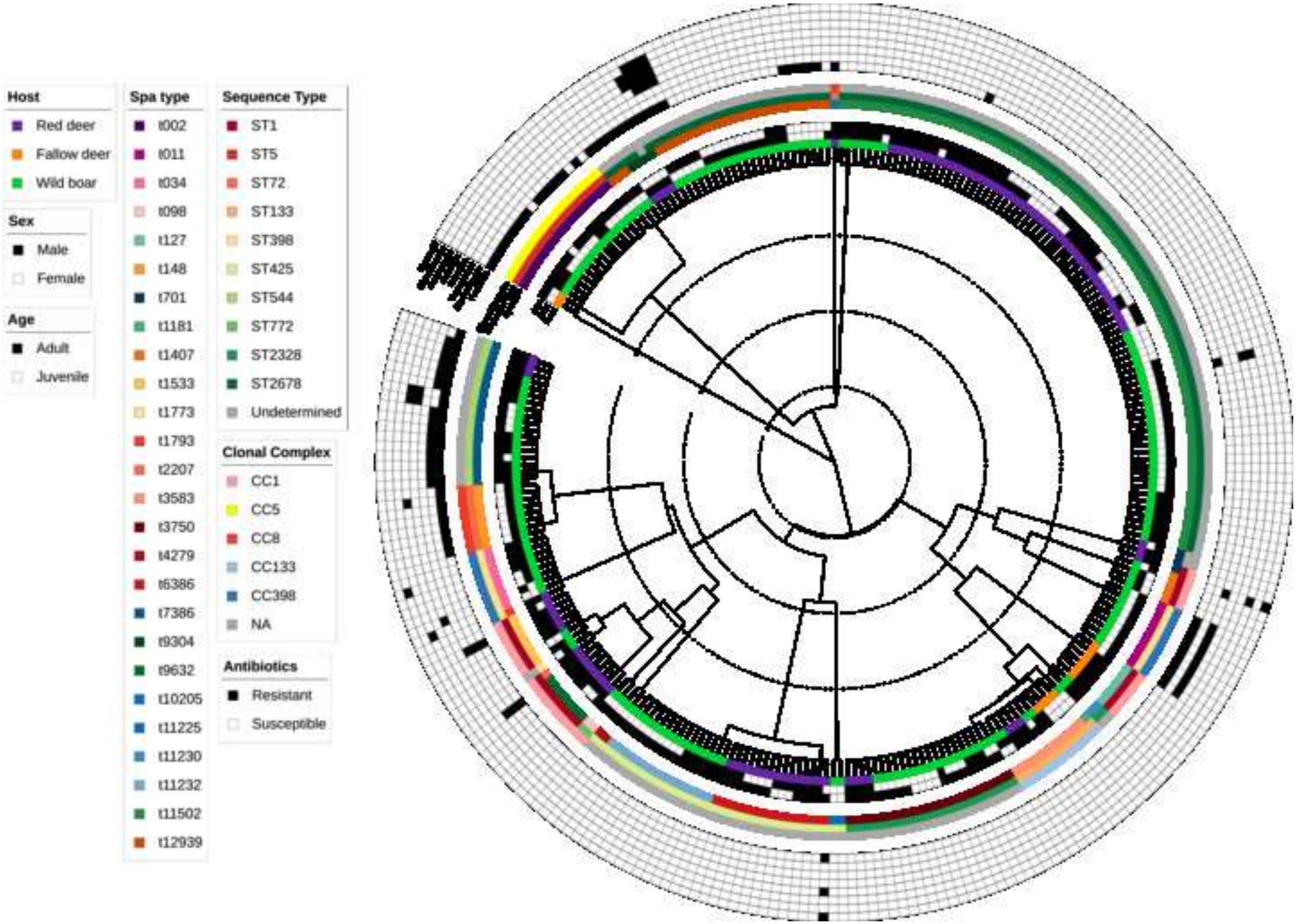

Figure 4

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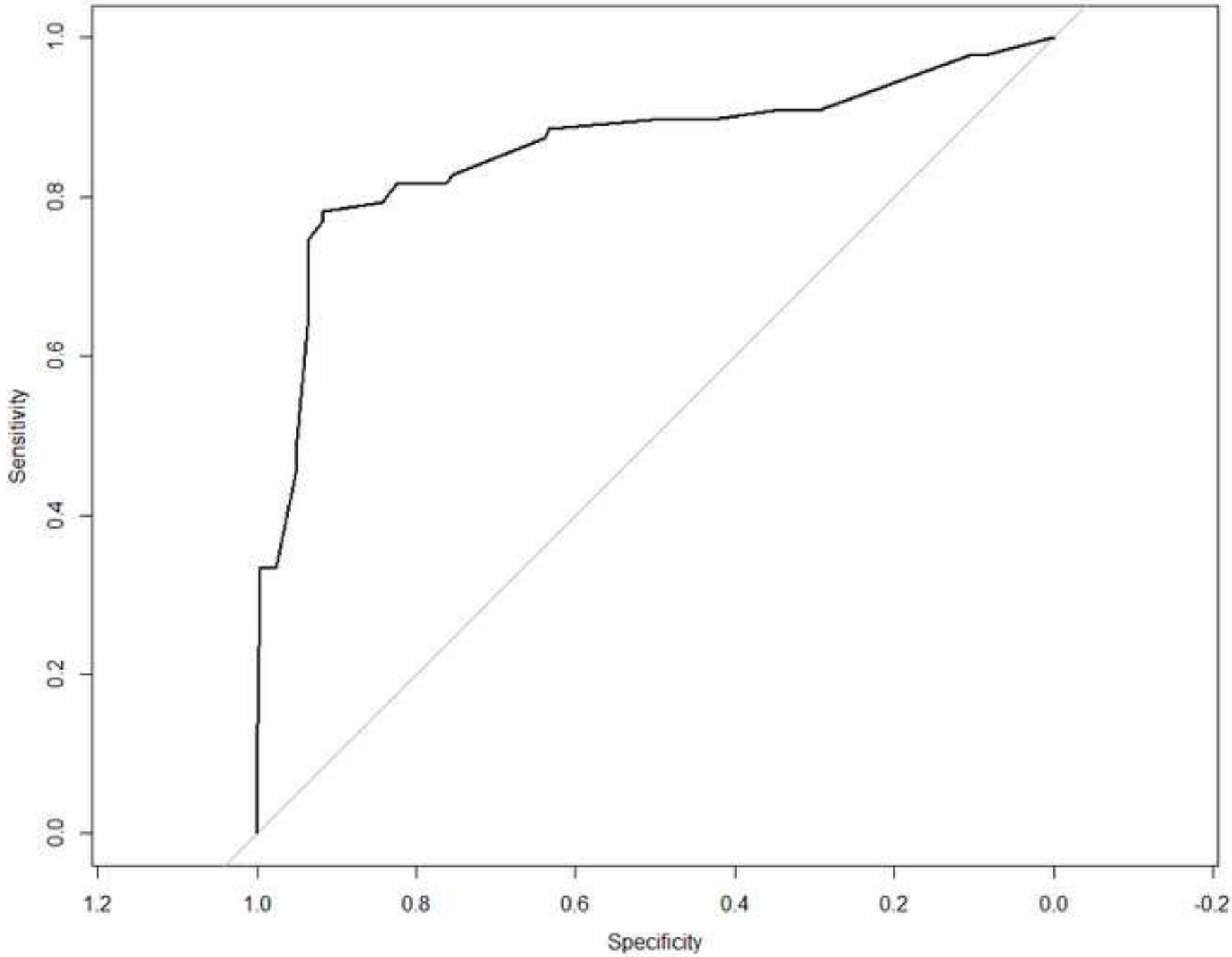

declaration of competing interests

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

AUTHOR STATEMENT

Mónica V. Cunha: Conceptualization; Methodology; Investigation; Supervision; Resources; Funding acquisition; Writing - original draft, review & editing**; Beatriz Ramos:** Experimental work; Formal analysis; Visualization; Writing - original draft; **Luís Miguel Rosalino:** Validation; Writing - review & editing; **Josman Palmeira:** Resources; **Rita Torres:** Funding acquisition; Writing - review & editing. All authors approved the final manuscript.

Antimicrobial resistance in commensal *Staphylococcus aureus* from wild ungulates is driven by agricultural land cover and livestock farming

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Keywords: *Staphylococcus aureus*; MRSA; MLST; wildlife; antimicrobial resistance; ecological modelling; human perturbation.

ABSTRACT

Staphylococcus aureus is a human pathobiont (i.e., a commensal microorganism that is potentially pathogenic under certain conditions), a nosocomial pathogen and a leading cause of morbidity and mortality in humans. *S. aureus* is also a commensal and pathogen of companion animals and livestock. The dissemination of antimicrobial resistant (AMR) *S. aureus*, particularly methicillin-resistant (MRSA), has been associated to its ability for establishing new reservoirs, but limited attention has been devoted to the role of the environment. To fill this gap, we aimed to characterize animal carrier status, AMR phenotypes, predominant clonal lineages and their relationship with clinical and food-chain settings, as well as to find predictors of AMR occurrence. Nasal swabs (*n*=254) from wild boar (*n*=177), red deer (*n*=54) and fallow deer (*n*=23) hunted in Portugal, during the season 2019/2020, yielded an overall carrier proportion of 35.8%, ranging from 53.7% for red deer and 32.2% for wild boar to 21.7% for fallow deer. MRSA from wild boar and phenotypically linezolid-resistant *S. aureus* from wild boar and red deer were isolated, indicating that resistance to antimicrobials restricted to clinical practice also occurs in wildlife. The most prevalent genotypes were t11502/ST2678 (29.6%) and t12939/ST2678 (9.4%), previously reported in wild boar from Spain. Clonal lineages reported in humans and livestock, like CC1, CC5 or CC8 (19.1%) and ST425, CC133 or CC398 (23.5%), respectively, were also found. The sequence type ST544, previously restricted to humans, is described in wildlife for the first time. We also identified that land use (agricultural land cover), human driven disturbance (swine abundance) and host-related factors (~~host~~ sex) determine resistance occurrence. These findings suggest that antibiotics used in clinical settings, agriculture and livestock farming, spill over to wildlife, leading to AMR emergence, with potential biological, ecological, and human health effects. This work is one of the most comprehensive surveys in Europe of *S. aureus* occurrence and determinants among widely distributed wild ungulates.

INTRODUCTION

Staphylococcus aureus is a pathobiont (i.e., a potentially pathogenic microorganism under certain circumstances), colonizing the nasal mucosa and skin of humans and, often, other animals, e.g., swine (Strube, Hansen, Rasmussen, & Pedersen, 2018). However, it is also associated to human disease, from skin or soft tissue infections to systemic and fatal illness (Sakr et al., 2018). While *S. aureus* has been most commonly reported as a human and nosocomial pathogen, it has been also isolated from a wide range of ~~host~~ wild species, mainly mammals and birds (Feßler et al., 2018; Schaumburg et al., 2012), in recent years. Additionally, it has been reported in environmental matrices, such as water and wastewater (Silva et al., 2020), air (Kozajda, Jeżak, & Kapsa, 2019), and soil (Johny, 2019).

The role of non-human hosts in *S. aureus* transmission is emphasized by the fact that most clonal complexes (CCs) include strains recovered from diverse ecological settings (Matuszewska et al., 2020). ~~One strong~~ A good example is the case of antimicrobial resistant (AMR) *S. aureus*, particularly methicillin-resistant (MRSA), which have acquired resistance to β-lactams, posing major public health concerns. MRSA have been recovered from numerous healthy and sick animal species, including livestock, companion animals (Monecke et al., 2016) and wildlife (Porrero, Mentaberre, et al., 2014; Ruiz-Ripa et al., 2019; Seinige, Von Altrock, & Kehrenberg, 2017). Dissemination of AMR *S. aureus* has been associated to its ability for establishing new reservoirs (Heaton et al., 2020). The ~~resolution~~ results obtained from molecular characterization has allowed to link particular CCs, like CC398 or CC133, to the animal component, namely livestock, ~~of~~ on *S. aureus* transmission (Hoekstra et al., 2020; Price et al., 2012). However, typical human CCs, like CC1, CC5 or CC8, have also been reported in livestock, raising concerns on host jumps (Heaton et al., 2020; Richardson et al., 2018).

Wildlife contact with xenobiotics and antimicrobial agents can happen either by indirect contact with contaminated environmental matrices or environmental pollution driven by

livestock farming and human activity, or from direct contact with livestock in aggregation points during feeding (Torres, Carvalho, et al., 2020). Animals colonized with AMR bacteria may spill-over bacteria or antimicrobial resistance genes (ARG) to conspecifics and other species (Plaza-Rodríguez et al., 2021; Torres, Fernandes, et al., 2020; Vittecoq et al., 2016). In recent years, reporting AMR isolates in wildlife is increasingly common (Monecke et al., 2013; Palmeira et al., 2021; Porrero, Mentaberre, et al., 2014; Ruiz-Ripa et al., 2019; Torres, Fernandes, et al., 2020). This fact might be justified, not only by an increasing interest in wildlife health research, but also by the increase of interspecific transmissions in the human-livestock-wildlife interface (Torres, Carvalho, Cunha, & Fonseca, 2019). Prophylactic and metaphylactic treatments of farmed animals are considered the main drivers of antimicrobial resistance in bacteria (Marshall & Levy, 2011). The intensive use of antimicrobials in agriculture or aquaculture that promote environmental contamination also exert downstream selective pressure upon microorganisms (Laxminarayan et al., 2013).

In Portugal, antimicrobial resistant *Staphylococcus* spp., or typically healthcare-associated strains (Nazareth et al., 2012; Peres, Pina, & Fonseca Cardoso, 2011), and MRSA, have been isolated from pet dogs (Silva, Oliveira, et al., 2021), wild rabbits (Sousa et al., 2020), wild rodents (Silva, Gabriel, et al., 2021) and wild boars (Sousa et al., 2017). Detection of *mecA* or *mecC* has also been reported (Silva, Gabriel, et al., 2021; Sousa et al., 2017). The molecular characterization of wildlife isolates indicates the circulation of strains from diverse CCs, such as CC1, CC5, CC8, or the livestock-associated CC133 or CC398 strains (Silva, Gabriel, et al., 2021; Sousa et al., 2017; Sousa et al., 2020). Particular attention has been given to clonal lineages widely spread in humans, livestock and companion animals, but information on the incidence of MRSA in the environment, particularly in wildlife, remains overlooked.

In this work, we aimed to extend previous knowledge on the occurrence of *S. aureus* in wildlife, namely in three species of wild ungulates, such as wild boar (*Sus scrofa*), red deer (*Cervus*

elaphus) and fallow deer (*Dama dama*), ~~that~~ which present contrasting ecology (e.g. feeding and space use strategies) and degree of synanthrophization (Torres, Fernandes, et al., 2020). We also aimed to disclose the drivers contributing for antimicrobial resistance among commensal bacteria or pathobionts from wild species that have limited or indirect contact with anthropogenic sources of antimicrobials (Torres, Fernandes, et al., 2020). For this purpose, we used *S. aureus* as a bacterial model, due to its ubiquity and wild ungulates as animal model species. Indeed, these species are affected by several factors that have impact their distribution and the way they use space, with repercussions in their ability to move and disperse AMR determinants. The specific aims of this work ~~were thus to~~are: 1) to characterize the animal carrier status for *S. aureus*; 2) to describe the molecular types and antimicrobial resistance profiles of *S. aureus* isolated from sampled hosts; 3) to investigate the predominant clonal lineages and their relationship with clinical and food-chain settings; and 4) to determine the impact of biotic and abiotic factors on the occurrence of AMR *S. aureus* ~~occurrence~~.

These aims could be reached by means of a large survey across different environmental contexts, taking advantage of biological specimen availability from hunted animals and ecological modelling, setting the scene for one of the most comprehensive studies in Europe of *S. aureus* occurrence among widely distributed wild ungulates, at the frontier of humanized landscapes.

METHODS

Sample collection

A total of 254 nasal swabs from three species of wild ungulates (wild boar, *n*=177; red deer, *n*=54; fallow deer, *n*=23) were collected during the 2019/2020 legal hunting season (from October 2019 to February 2020) across mainland Portugal. Animals were sampled, and nasal specimens were collected within 1-5 h after death. Nineteen different sampling sites were

included in this study (Supplementary Figure 1), encompassing a high diversity of land cover (illustrated in Supplementary Figure 2) and also capturing different animal densities. Host species, sex and age class were registered for each sampled animal.

Selective isolation of *Staphylococcus aureus*

For selective isolation, all nasal swabs were subjected to selective enrichment in Mueller-Hinton medium (Sigma, Merck KGaA, Germany) supplemented with 6.5% NaCl, to select for the characteristic halotolerance of clinically-relevant *Staphylococcus* sp., namely *S. aureus*. After 18-24 hours incubation at 35ºC±1ºC, each pre-enriched sample was plated (100 µl) onto Mannitol Salt Agar (MSA) (Oxoid, Thermo Fisher Scientific, USA) and colonies suggesting ~~presenting differential colour suggestive of~~ mannitol fermentation, and thus hypothesized as *S. aureus*, were selected from each plate. Up to eight colonies with distinctive morphology or size from each plate, grown from each enrichment in MSA, were sub-cultured onto Nutrient Broth Agar (Biokar, Solabia, France). All isolates retrieved from MSA were tested for β-haemolysis in Columbia agar medium with 5% sheep blood (bioMérieux Clinical Diagnostics, France). The previous protocol was adapted from the procedure described in "Isolation of methicillin-resistant *Staphylococcus aureus* (MRSA) from food-producing animals and farm environment" from the European Food Safety Authority (EFSA) (European Food Safety Authority, 2012). A quality control strain (*Staphylococcus aureus* ATCC 25923) was used to interpret and validate each test batch.

All isolates that tested positive for β-haemolysis were subjected to molecular identification as *S. aureus* through polymerase chain reaction (PCR), performed according to Martineau and collaborators (Martineau et al., 1998), by means of two *S. aureus*-specific primers and an additional set of 16S rRNA primers for internal amplification control (Marchesi et al., 1998). The full characterization of each *S. aureus* isolate is described in Supplementary Table 1.

Molecular amplification of *spa* hypervariable region and Multi-Locus Sequencing Typing

The molecular amplification of the *spa* hypervariable region was performed by PCR using the primers described by Stegger and collaborators (Stegger et al., 2012). Multi-locus sequencing typing (MLST) (Enright et al., 2000) was performed for a single random isolate representative of each retrieved *spa* type, comprising a total of 20 isolates representing 19 *spa* types. Strain *Staphylococcus aureus* ATCC 25923 was used as positive control in all PCR assays. A no-template control with nuclease-free water was also added in each PCR assay.

The *spa* amplicons and MLST fragments were sequenced and assigned using *spa* typing and MLST plugins, respectively, from BioNumerics v6.6 (Applied Maths).

The discriminatory power (D) of each technique, i.e. the average probability that the typing method will assign a different type to two unrelated isolates sampled in the population, was calculated based on Simpson's index of diversity (Hunter & Gaston, 1988) using the online tool "*In silico* simulation of molecular biology experiments" (Bikandi, Millán, Rementeria, & Garaizar, 2004). The individual allelic diversity (h) of the *loci* from MLST scheme tested were obtained in a similar manner.

Phylogenetic analysis

For the phylogenetic analysis, one minimum spanning tree was computed with results from *spa* typing as input, using BioNumerics v6.6 (Applied Maths). The advanced cluster analysis was performed using the categorical data as a similarity coefficient, and calculated a standard minimum spanning tree with single *locus* and double *loci* variance priority rule. The dendrogram was computed using *spa* fragment sequences as input using BioNumerics v6.6 (Applied Maths) calculating Pearson correlation coefficient, and the unweighted pair group method with arithmetic means (UPGMA) as the agglomerative clustering algorithm. The annotation was performed using Interactive Tree Of Life (iTOL) v5 (Letunic & Bork, 2021).

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed by disk-diffusion and broth microdilution methods, according to the guidelines of the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2021). The following antimicrobial agents were tested by disk-diffusion (µg per disk): benzylpenicillin (1 unit), cefoxitin (30), ciprofloxacin (5), erythromycin (15), gentamicin (10), tetracycline (30), linezolid (30) and trimethoprim-sulfamethoxazole (1.25 + 23.75). The broth microdilution assays were performed for oxacillin and vancomycin, according to EUCAST guidelines (EUCAST, 2021). Briefly, a McFarland 0.5 suspension of all isolates was used to inoculate Mueller-Hinton broth or Mueller-Hinton Agar (Oxoid, Thermo Fisher Scientific, USA), for broth microdilution and disk diffusion methods, respectively, and incubated overnight at 37°C. This panel of antimicrobials represent eight antimicrobial classes: aminoglycosides (gentamicin), β-lactams (oxacillin, cefoxitin and benzylpenicillin), fluoroquinolones (ciprofloxacin), folate pathway inhibitors (trimethoprim-sulfamethoxazole), glycopeptides (vancomycin), macrolides (erythromycin), oxazolidinones (linezolid) and tetracyclines (tetracycline), and thus the most frequent cellular targets, namely cell envelope, protein synthesis at both 30S and 50S ribosomal subunits and nucleic acids biosynthesis. All antimicrobial susceptibility tests were evaluated according to EUCAST clinical criteria, as we intended to link the retrieved isolates with the potential spill-over of human-associated isolates into the environment. The genotypic resistance profile to β-lactams was evaluated based on the amplification of *mecA* and *mecC* gene markers, according to Stegger and colaborators (Stegger et al., 2012). Multidrug resistant isolates were defined according to Magiorakos and co-workers (Magiorakos et al., 2012): resistance to at least one antimicrobial from at least three different antimicrobial classes or identification as MRSA through molecular detection of either *mecA* or *mecC* gene markers.

Statistical analyses

198 Statistical analyses were performed using non-parametric Mann-Whitney test for independent
199 and continuous variables in order to evaluate differences in carrier proportion across host, sex,
200 and age. The *p* values were determined and discussed accordingly using α=0.05 to reject the
201 null hypothesis (considered significant if $P < 0.05$*)*. The 95% confidence intervals (CI) were also
202 estimated. Multivariate analyses, namely Principal Components Analysis (PCA), were
203 performed for the top five most frequent molecular types and for all classes of resistance
204 phenotypes (i.e. multidrug resistant, resistant and susceptible), using "prcomp" function from
205 R software v4.0.5 and the threshold established for relevant association was ±0.2. The
206 exploratory approach was performed with the following variables: sampling district (Beja,
207 Évora, Portalegre and Setúbal [Alentejo], Coimbra, Leiria, Guarda [Central Portugal], Porto
208 [North Portugal]); sex (male and female); age (adult and juvenile); host species (wild boar, red
209 deer and fallow deer).

210 Geographic information was used at different scale levels (NUTS II statistical regions, district
211 administrative region, municipality administrative region) whenever appropriate.

212 **Modelling procedures**

213 Having in mind the ecology of wild ungulates and the factors that may facilitate exposure to
214 antimicrobials, and thus impact the emergence of AMR bacteria and/or the exchange of AMR
215 determinants, we explored the effect of environmental, host-related, land use, human driven
216 disturbance factors or spill-over from livestock on AMR occurrence. To test these hypotheses,
217 we examined 17 variables as putative predictors of AMR occurrence. For this analysis, six
218 categories of variables were considered, representing different working hypothesis, as
219 described in Table 1. All variables were considered at the Municipality level due to the lack of
220 the exact geographical coordinates of the location where each animal was hunted. Land use
221 data was retrieved from Carta de Uso e Ocupação do Solo (2018) at the National Geographic
222 Information System [Sistema Nacional de Informação Geográfica (SNIG)] (Direção Geral do

Território - Portugal, 2018). Livestock abundance (2019) (INE, 2019) and population density (2020) (INE, 2020) were gathered from Instituto Nacional de Estatística (INE). Wild ungulates presence was defined based on the Atlas of Mammals in Portugal (Bencatel J. et al., 2019). Climate data comprised variables gathered from BioClim (2017) at 30 arc-second resolution (Fick & Hijmans, 2017). Both host sex and age class data were encoded as binary, where female and juvenile are encoded by zero and male and adult are encoded by one.

Regarding the modelling procedure, initially the data was tested for spatial autocorrelation to account for the degree of the geographical dependency of data, using the Moran's I index with the R's "ape" package (Paradis & Schliep, 2018), using the municipality centroid as a surrogate of the animal's hunting location. Multicollinearity was tested by estimating the Variation Inflation Factor (VIF) between variables within the same hypothesis, using R's "corvif" function from source code "HighstatLibV10" (Zuur, Hilbe, & Ieno, 2013). Variables with an estimated VIF above five were discarded from analysis, being that value considered a cut-off for excessive inflation in variance caused by the existing multicollinearity (Zuur et al., 2013). All the remaining candidate variables were standardized prior to modelling procedure for straightforward results' analysis. We applied a Generalized Linear Mixed Model (GLMM) approach to identify the drivers shaping antimicrobial resistance presence/absence variation. GLMM were built using a logit link function applied to a binomial distribution, and considering municipality as a random effect due to the presence of spatial autocorrelation of data (F. Dormann et al., 2007). Such modelling procedures were applied using the "lme4" package in R v.4.0.5 (Bates, Mächler, Bolker, & Walker, 2015).

In this analysis, a two-folded analytical approach was implemented. First, only the variables associated to each specific working hypothesis were tested, and the models corresponding to all combinations of those variables were built. Then, an Information Criteria approach (Akaike Information Criteria -AICc - corrected for small samples) was used, in other to perform model

selection for each hypothesis. All produced models for each hypothesis were ranked according with their ΔAICc (corresponding to the difference between each model AICc and the smaller AICc value), with models presenting ΔAICc<2 being retained as best models. The best model(s) for each hypothesis were compared using the AIC approach to test which hypothesis was more supported by the data (i.e. the model with the lowest AICc).

In the second step, we selected the variables included in the best model for each hypotheses that showed a 95% confidence interval that did not cross zero (i.e. informative variables for which is possible to assess the effect on the dependent variable – positive vs negative; (Arnold, 2010)). Those variables were used to test a combined hypothesis, which defended that antimicrobial resistance variation is driven by multiple origin factors. As more than one model fulfilled the best model criterion (i.e., ΔAICc<2), a model averaging procedure was implemented to estimate variables coefficients. Model selection procedures were implemented in R using "MuMIn" package (Barton, 2015). Best model validation was tested by the Receiver Operating Characteristic (ROC) Curve and the Area Under the Curve (AUC), using the R package "pROC" (Robin et al., 2011).

RESULTS

Staphylococcus aureus carrier status among wild ungulates

The selective culture of nasal swabs (*n*=254) from hunted wild boar (*n*=177), red deer (*n*=54) and fallow deer (*n*=23), followed by molecular identification of presumptive *S. aureus,* yielded 300 methicillin-susceptible *S. aureus* isolates, along with three MRSA isolates. The overall proportion of *S. aureus* carriers (i.e. confirmed *S. aureus* isolation in each host) was 35.8% (95% CI: 29.89-41.76%). The overall proportion of carriers by host was established at 53.7% (95% CI: 40-67.4%) for red deer and 32.2% (95% CI: 25.3-39.2%) for wild boar (Table 2). As for fallow

deer, samples were collected in one site only, in the Southern region, with an overall proportion of carriers of 21.7% (95% CI: 3.5-40%) (Table 2).

Different regions (NUTS II) of Portugal presented differences in the overall proportion of carriers. The Central region was identified as hosting significantly more *S. aureus* carriers than the North and Alentejo (Supplementary Figure 1). The Central region (Mean: 58.14%; 95% CI: 47.5-68.78%) was identified as hosting significantly (Mann-Whitney test, α=0.05; p<0.001) more *S. aureus* carriers than the North (Mean: 14.29%, 95% CI: 0-30.6%) and Alentejo (Mean 26.09%; 95% CI: 18.67-33.51%). For red deer, there was no statistical differences (Mann-Whitney test, α=0.05; p-value = 0.1542) when comparing the proportions between male and female hosts (male: 61.1% [95% CI: 44.4-77.8%]; female: 38.9% [95% CI: 13.9-63.8%]), with a p-value of 0.1542 (Mann-Whitney test), and also when comparing juveniles and adults (juveniles: 70% [95% CI: 35.4-100%]; adults: 50% [95% CI:34.6-65.4%]), with a p-value of 0.3095 (Mann-Whitney test, α=0.05). Similarly, wild boar did not show any statistical differences regarding the proportion of carriers among male and female hosts (male: 32.4% [95% CI: 21.5-43.3%]; female: 32% [95% CI: 22.8-41.2%]) (Mann-Whitney test, α=0.05; p-value=1). Only the carrier proportion of wild boar juveniles and adults were significantly different (juveniles: 22.4% [95% CI: 12.1-32.6%]; adults: 38.2% [95% CI:28.9-47.4%]) (Mann-Whitney test, α=0.05; p-value=0.0320).

Molecular diversity of *Staphylococcus aureus* from wild ungulates

The *spa* typing analysis was successfully applied to 277 out of *S. aureus* 303 isolates. We repeated PCR analyses for the non-typeable 26 isolates testing different sets of reactional and thermal profiling conditions, but *spa* amplification for those isolates was unfortunately not achieved, even though molecular identification as *S. aureus* was confirmed by PCR. The 277 typeable isolates yielded 26 *spa* types (Table 2), the most predominant (top five) being t11502 (n=82 isolates), t12939 (n=26), t3750 (n=22), t7386 (n=18) and t002 (n=17) (Figure 1). The

297 presence of nine isolates from t011 and eight isolates from t034, usually associated to
298 livestock, was also registered in wild boar and fallow deer. The analysis of the distribution of
299 *spa* types per host species evidenced that both wild boar and red deer share t11502 as the
300 most frequent *spa* type. From the top five, wild boar isolates represent all the isolates from
301 t12939 (*n*=26) and also account for the majority of isolates from *spa* types t11502 (*n*=37), t3750
302 (*n*=18), t7386 (*n*=15), and t002 (*n*=15). As for red deer, all isolates from t6386 (*n*=16) were
303 retrieved from this host species, while fallow deer isolates were characterized by three *spa*
304 types only, namely t127 (*n*=6), t3583 (*n*=4) and t002 (*n*=2). Since up to 8 colonies with
305 distinctive morphology were peaked from each swab enrichment in mannitol salt agar for
306 further characterization, we registered that among the 254 animals surveyed, only twelve
307 harboured more than one *spa* type *S. aureus*. These six wild boar and six red deer correspond
308 to 11% and 21% of overall wild boar and red deer *S. aureus* carriers, respectively.

309 An exploratory approach to analyse the association of *spa* types with the available variables
310 (see methods) showed that the first two principal components (PCs) accounted for 52.9% of
311 the variance (Supplementary Table 2). Host species and sex variables were retained by the first
312 two components, with similar eigenvalues, i.e., with the variance being similarly explained
313 within both components. The variables that were more related to PC1 were thus sex, host
314 species (wild boar and red deer) and the sampling district (Leiria) (Supplementary Figure 3). As
315 for PC2, the most related variables were sex, age class, host (wild boar and red deer) and
316 sampling district (Beja) (Supplementary Figure 3). Members of *spa* type t7386 cluster together
317 in the positive axis of PC2, being more related to the variables male, adult, wild boar, and Beja
318 sampling district (Supplementary Figure 3). All those relations were found to be significant
319 (Supplementary Table 3) except for wild boar (*p*-value = 0.1832). This host species does not
320 seem to carry significantly more isolates belonging to t7386 than the remaining. Similarly,
321 members of *spa* type t002 and t12939 cluster together on the positive axis of PC1, being more
322 related to the variables female, wild boar, and Portalegre and Guarda sampling districts

(Supplementary Figure 3). While for *spa* type t002, none of the variables indicated by the exploratory analysis revealed significant differences (i.e., p>0.05). For *spa* type t12939, all variables, with the exception of sex, showed a significant influence, and a negative relation of Guarda district with this *spa* type was highlighted (*p*-value = 0.0156) (Supplementary Table 3). The *spa* type t11502 was more frequent in the Central region, even though its presence was noted in almost all sampled districts (Figure 2). Similarly, *spa* type t6386, which was exclusively found in red deer, is more frequent in the Central region as well, since this host is more represented in this region (Figure 2).

Since *spa* type/sequence type pairs are very frequently linked, with most *spa* types being associated to a particular sequence type, MLST analysis was applied to 20 isolates (nine from wild boar, eight from red deer, and three from fallow deer) representing the diversity of the most common *spa* types (i.e. over 1% frequency). These 19 *spa* types were grouped into ten different STs. The presumptive ST of the remaining isolates was inferred from the ST of the representative isolate within each *spa* type, similarly to what has been previously adopted in several works (Porrero, Mentaberre, et al., 2014; Ruiz-Ripa et al., 2019). Thus, the most common STs were presumably ST2678 (*n*=112), ST425 (*n*=31), ST1 (*n*=25), ST2328 (*n*=24), ST544 (*n*=18) and ST398 (*n*=17) (Table 2). Of the ten STs, only ST772 was found exclusively in wild boar, with the remaining ST being shared by two or three host species. Fallow deer was found to carry members of ST1, ST133 and ST5. Red deer hosted members of ST2678, ST425, ST1, ST2328, ST133, ST398, ST72, and ST544. Wild boar hosted isolates from each sequence type found. The ten STs detected cluster into five CCs, of which three are strongly associated to human infection (CC1, CC5 and CC8), while two are related with infection in livestock (CC133 and CC398). According to the multivariate analysis performed to examine the association of STs with the available variables, the first two PCs accounted for 51.4% of the variance (Supplementary Table 2). The variables that were more related to PC1 were sex, host (wild boar and red deer) and the sampling district (Leiria) (Supplementary Figure 4). As for PC2, the

more related variables were sex, age class and host (wild boar and red deer) (Supplementary Figure 4). When clustered according to STs in the PCA, isolates from ST544 were the only ones presenting a restricted association with variables male, adult, and wild boar, while the remaining top 6 STs presented a wider distribution in the first two PCs (Supplementary Figure 4). Both adult trait and wild boar as host species were correlated with ST544 (Supplementary Table 3). Sequence types do not appear to present any geographical bias (Supplementary Figure 5).

The overall discriminatory power of both techniques was high, with *spa* typing (D=0.8774) presenting higher power than MLST (D=0.7879). Although the number of isolates subjected to *spa* typing was higher than those effectively submitted to MLST, it should be reminded that the discriminatory power accounts for the number of types and their relative abundance. Concerning allelic diversity of each MLST *locus,* the values ranged from 0.6903 to 0.7865, with *yqiL* being the most discriminatory: *pta* (h=0.6903), *glpF* (h=0.7098), *tpi* (h=0.7273), *arcC* (h=0.7355), *gmk* (h=0.7562), *aroE* (h=0.7614) and *yqiL* (h=0.7865).

Antimicrobial resistance phenotypes and genotypic resistance markers

Concerning antimicrobial resistance, the phenotypic characterization of 303 isolates led to the identification of 91 *S. aureus* resistant to at least one antimicrobial agent according to EUCAST clinical criteria~~,~~ . ~~with ten of those~~From those, ten were ~~being~~ classified as multidrug resistant, three being positive for the detection of *mecA* resistance marker and the remaining presenting a non-wild type phenotype to at least one antimicrobial agent from at least three different categories (Table 3). The *mecC* marker was not detected among the 303 *S. aureus* isolates. All multidrug resistant isolates, with the exception of one isolate from red deer, were retrieved from wild boar (Table 2). Additionally, one isolate exhibited a MIC of 4 mg/L for vancomycin, which although being considered wild-type (i.e. susceptible) according to EUCAST clinical criteria (EUCAST, 2021), stands right at the MIC breakpoint value above which resistance (non-

wild type) is considered (Table 3). The antimicrobials to which phenotypic resistance was more frequent were penicillin (27.1% of isolates), followed by erythromycin (6.9%), tetracycline and cefoxitin (4.3%), ~~gentamycin~~ gentamicin and oxacillin (3.3%), ciprofloxacin (1.7%) and ~~finally~~ linezolid (1.0%) (Supplementary Table 1). In the whole set of 303 isolates, resistance to either trimethoprim-sulfamethoxazole or vancomycin was not detected. Isolates from different hosts presented different patterns of resistance: for cervids, resistance to benzylpenicillin (25.9%), tetracycline (7.4%), oxacillin (3.7%), cefoxitin (1.9%) and ciprofloxacin (0.9%) was registered. The isolates from wild boar presented resistance to all antimicrobials from the selected panel, with the exception of trimethoprim-sulfamethoxazole and vancomycin, as referred above. Principal component analysis of resistance phenotypes categories was completed: isolates were classified into three categories according to their antimicrobial resistance phenotype: "Susceptible" for fully susceptible isolates, "Resistant" for isolates resistant to at least one antimicrobial agent and "Multidrug resistant" for isolates defined as such, according to the definition ~~presented up above~~previously defined. The first two PCs account for 50.9% of the variance (Supplementary Table 2), showing that the variables more related to PC1 were sex, host (wild boar and red deer) and the sampling district (Leiria) (Supplementary Figure 6). As for PC2, the most related variables were sex, age class and sampling district (Portalegre and Leiria) (Supplementary Figure 6). When clustered, multidrug resistant (MDR) isolates exhibited a restricted association to variables adult, female and wild boar (Supplementary Figure 6), with a strong correlation of MDR to the female trait supported by a statistical test (*p*-value = 0.0005) (Supplementary Table 3). The highest number of antimicrobial resistant isolates were retrieved from hosts in sampling site number 33, located in Beja district (*n*=29), sampling site number 21, located in Setubal district (*n*=13) and sampling site number 19, in Portalegre district (*n*=14).

Results from molecular typing and antimicrobial resistance are summarized in Figure 3.

Ecological modelling to unravel significant predictors of AMR occurrence

399 The data presented spatial autocorrelation (Moran's I = 0.057; *p*-value < 0.001) indicating the
400 need to use the variable Municipality as a random effect in the GLMM, to account for this data
401 structure. Multicollinearity was confirmed for variables Forest_cov, Cattle_abund and
402 Driest_prec, which were then discarded from the corresponding hypothesis due to the high
403 VIF values (VIF>5). Thus, the combinations of the following variables were tested for each
404 hypothesis: H1) Land Use, H2) Livestock abundance, H3) Anthropogenic disturbance, H4) Wild
405 ungulates presence, H5) Life-history traits and H6) Climate. The hypothesis with the highest
406 support was the combined hypothesis, which registered lowest overall AICc (Table 4).
407 However, four models within this hypothesis showed a ΔAICc<2, and therefore we estimated
408 an average model representing the most parsimonious and explanatory hypothesis to describe
409 the presence/absence of antimicrobial resistance in *S. aureus* isolates. Those four models were
410 composed by a combination of four variables: Agriculture, Sex, Swine and Mouflon. The
411 average model highlights the influence of Agricultural land cover, swine abundance and sex as
412 the predictors of antimicrobial resistance, with 95% confidence intervals that did not cross
413 zero. Thus, areas with higher proportion of agricultural lands and swine abundance have a
414 higher probability of antimicrobial resistance occurrence (Supplementary Table 4).
415 Furthermore, females were also more prone to host antimicrobial resistance strains than
416 males, supporting the preliminary predictions by the use of PCA (Supplementary Table 4). The
417 AUC value derived from the ROC curve reached 0.8601, revealing good accuracy of the average
418 model to predict the occurrence of antimicrobial resistance in *S. aureus* isolates (Figure 4)
419 (Manel, Williams, & Ormerod, 2001).

420

421 **DISCUSSION**

422 The global dissemination of ARG and AMR bacteria presents a global threat to public and
423 ecosystem health. Most studies on *S. aureus* have focused the human and veterinary settings,
424 while the environmental sphere, including wildlife, has been neglected. However, to

425 understand the ~~life cycle~~circulation of antimicrobial resistance determinants, including those
426 specifically associated to *S. aureus* ecology, it is the key to expand surveillance at the interface
427 of natural and humanized environments. Wild ungulates offer that opportunity, which was
428 thus explored in this study. They may establish the link between different environments (e.g.
429 urban, natural and semi-natural) through a complex web of interactions, as they have large
430 home ranges, are untreated with antibiotics, and their habitat can overlap with livestock and
431 humans (Torres, Fernandes, et al., 2020). Furthermore, distinctive ecological features (e.g.
432 feeding habits, space use) and life history traits, like the foraging and opportunistic feeding
433 behaviour of wild boar near human settings, which contrasts with human avoidance among
434 deer, makes them good models to assess anthropogenic influence on AMR dissemination. Our
435 results show that wild ungulates carry *S. aureus*~~,~~ at a relatively high proportion~~, *S. aureus*~~.
436 These results are in line with others across Europe (Monecke et al., 2016; Porrero, Mentaberre,
437 et al., 2014; Ruiz-Ripa et al., 2019; Seinige et al., 2017). They show different carrier proportions
438 among the different surveyed host species, but also highlight geographical differences. In this
439 work, there is a higher representability of the Central to South region of Portugal, less explored
440 so far in the few existing studies. Differences in the overall proportion of carriers between
441 regions was verified.~~.~~ Interestingly, red deer is also more abundant at the Centre. The
442 estimated carriage percentages point to a proportion of 32.2% carriers amongst wild boar in
443 mainland Portugal. Likewise, in Spain, the percentage of carriers has been reported to range
444 from 17.7% to 65.0%, depending on the surveyed geographical area, with the first work
445 including ten different Spanish provinces, while the later was focused on one region only
446 (Porrero, Mentaberre, et al., 2014; Ruiz-Ripa et al., 2019). Other European countries like
447 Germany, Austria or Sweden have also reported wild boar as a carrier of *S. aureus* (Monecke
448 et al., 2013; Seinige et al., 2017), highlighting the role of this species as a maintenance host. As
449 for red deer, we found that 53.7% were carriers of *S. aureus*, which is higher than the average
450 rates reported in Spain for red deer under semi-extensive farming, ~~which range~~ranging from

24.6% to 44% (Gómez et al., 2015; Ruiz-Ripa et al., 2019). As for wild red deer, the reported values in other countries range from 19.78% in Spain to 90.67% in Italy (Luzzago et al., 2019; Porrero, Mentaberre, et al., 2014). The latter prevalence rate represents an extreme scenario, in which an active culling program was applied thereafter due to overabundance of red deer in a specific region. Both wild boar and red deer have been reported to be increasing their population density across Europe, with the Iberian Peninsula reflecting such trend (Massei et al., 2015). The higher population density and changes in landscape cover have been possibly promoting the contact of wild ungulates with *S. aureus*, namely through the presence of wild boar, for which the foraging and feeding behaviour may promote a closer contact with human populations. Altogether, these studies suggest that *S. aureus* carriage in wild red deer in Portugal may be experiencing an increasing trend as well, along with host population density. Red deer are gregarious species, living in family groups, particularly the females, which could thus have spill over effects in other sympatric species (Palomo, Gisbert, & Blanco, 2007). Regular monitoring should thus be assured in the future.

Concerning the molecular characterization of the 303 *S. aureus* isolates, the most frequent *spa* types were t11502 (*n*=82), t12939 (*n*=26), t3750 (*n*=22), t7386 (*n*=18) and t002 (*n*=17). We also found one of the most frequent *spa* types across Europe, *spa* type t3750. This molecular type has been reported as the most frequent in wild boars in Spain (Porrero, Mentaberre, et al., 2014) and, more recently, in Portugal (Sousa et al., 2017). Correspondently, t11502/ST2678 and t12939/ST2678 presumably represent around one third of the total set of isolates retrieved, being previously reported only in Spain ~~only~~ (Heaton et al., 2020; Porrero, Mentaberre, et al., 2014). Also strongly represented was ST425, more commonly found in animals, ranging from small mammals, like rabbits, *Oryctolagus cuniculus* (Vancraeynest et al., 2006), or badgers, *Meles meles* (Monecke et al., 2016), to wild boars (Porrero, Mentaberre, et al., 2014; Porrero, Valverde, et al., 2014; Seinige et al., 2017), red deer (Monecke et al., 2016), roe deer, *Capreolus capreolus* (Porrero, Valverde, et al., 2014), or Iberian Ibex, *Capra pyrenaica*

(Porrero, Mentaberre, et al., 2014). In contrast, we also detected the presence of STs clustered in CCs like CC1, CC5 or CC8, which are more commonly associated to humans. However, those STs have also been found associated with particular animal hosts, like CC5 with poultry (Nübel et al., 2008) or CC8 with bovines (Resch et al., 2013), via host species jump events. In Portugal, the genetic diversity of *S. aureus* isolates from different hosts was evaluated in a study that reported humans hosting *S. aureus* belonging to CC22, CC5, CC15, CC8, CC398, CC30 and CC45 (Salgueiro et al., 2020) strains. As for the animal component, CC5, CC1, CC398, CC30, ST130 and ST121 strains were isolated (Salgueiro et al., 2020). We also found some of those molecular types in this work, namely CC1, CC5, CC8 and CC398. It should be noted that in the previous study, CC8 was exclusively linked to humans, while in our work we identified CC8 in wild ungulates as well. Such molecular diversity in wildlife raises questions on the origin of those strains, and on how sampled hosts may have interacted with livestock or humans.

Three MRSA isolates encoding *mecA* and belonging to *spa* type t7386/ST544 were detected. The set of 303 isolates was also tested for the presence of *mecC* resistance determinant, but none of the retrieved isolates presented amplification of this gene. Nevertheless, this determinant was previously linked to CC130 and ST425 (García-Álvarez et al., 2011), with the latter being one of the most frequent molecular types in this study. This finding may also be linked to the fact that *mecC* appears to have a low prevalence, even amongst animals, as estimated in a meta-analysis from 2016 that establishes *mecC* prevalence at 0.10% (Diaz, Ramalheira, Afreixo, & Gago, 2016). Additionally, we found discordant results concerning the lack of *mec* amplification in cefoxitin resistant isolates by agar disk diffusion, for which we were not able to clarify the source. To further explore the genetic region around *mecA*, whole genome sequencing will be applied to these isolates in the near future ~~work~~. The results of antimicrobial susceptibility testing to selected antimicrobials revealed that around one third of retrieved isolates were non-wild-type. Benzylpenicillin was the drug to which most isolates were resistant, with almost one third of 303 isolates exhibiting a non-wild-type phenotype, in

agreement with previous studies from Europe and northern Africa (Gómez et al., 2015; Mairi et al., 2019; Porrero, Mentaberre, et al., 2014; Sousa et al., 2017). Three phenotypically linezolid-resistant isolates were detected in wild boar and red deer. In agreement, linezolid-resistant *S. aureus* was recently reported in the North of Portugal and amongst healthy pigs (Leão, Amaro, Albuquerque, & Clemente, 2021; Sousa et al., 2017). The increasing description of linezolid-resistant *S. aureus* amongst wild and farmed animals has suggested that the use of antimicrobials in the treatment of human infections, leads to consequences that exceed widely the healthcare setting, as it is the case of MRSA (Kang et al., 2020; Sousa et al., 2017). This trend in the animal counterpart has followed the increasing pattern of usage of linezolid in the treatment of human MRSA infections in recent years (Matrat et al., 2020).

Although one third of *S. aureus* herein retrieved were resistant to one antimicrobial and some showed phenotypic resistance to linezolid and/or cefoxitin, very few were MDR, suggesting that most of these antibiotic resistance phenotypes may revert in the absence of antibiotic pressure. To mitigate these selective pressures, xenobiotic containment buffers across the environment are crucial. Most antimicrobial resistant isolates presumably belong to ST544 (*n*=18), ST2678 (*n*=18), ST5 (*n*=14), based on the inference of presumptive ST from the corresponding *spa* type representative. Those STs have been generally associated with human settings, like community or healthcare (Lin et al., 2017; Ochoa et al., 2020; Ruffing et al., 2017), highlighting the potential acquisition of antimicrobial resistance determinants by direct or indirect (Matuszewska et al., 2020) contact with humans. This contact may indeed be unexpectedly relevant since ST544, which harbours the higher number of the antimicrobial resistant isolates in our study, had never been previously reported in livestock or wildlife to the extent of our knowledge.

Potential interactions between wildlife and livestock may explain the presence of several molecular types associated to livestock amongst the wildlife animals sampled in this study. In

Portugal, wild boar and red deer are the main big game species, with free-ranging populations, but also may be maintained in high densities in fenced estates, ~~where cattle farming coexists~~coexisting with cattle farming (Gonçalves, Alcobia, Simões, & Santos-Reis, 2012). With generalist omnivorous behaviour, wild boar opportunistically feed on crops on agricultural lands, providing opportunities for indirect exposure to humans and human activities, and indirectly contact with livestock at aggregation spots for feeding and watering (Johann, 2020; Miller, 2017). Similarly, red deer seems to adapt its behaviour and habitat use when in direct contact with humans (Coppes, 2017). Many recreational areas, such as hunting grounds, and human forested areas overlap geographically with red deer's habitat, promoting temporary avoidance of areas with frequent human presence (Coppes, 2017). Nonetheless, red deer is suggested to develop habituation to human presence, mainly in regions where they are not hunted (Coppes, 2017). Most interactions between livestock and wild ungulates are found to be indirect through the partitioned use of resources (Carrasco-Garcia et al., 2016).

Artificial feed and water supplies devised for livestock in pastures and within farms can be particularly attractive for wildlife, fostering direct and indirect species interactions which is the case of Alentejo region (Carrasco-Garcia et al., 2016). Supplementary feeding and baiting in hunting estates are also common practices, increasing anthropogenic influence exerted upon wildlife (Laguna et al., 2021). Simultaneously, in North and Central Portugal, extensively managed livestock frequently share aggregation points, namely feed and water supplies, with wild ungulates, promoting indirect contacts and opportunities for spill over. In agreement with these notions, the percentage of isolates retrieved from wildlife that share molecular types more commonly associated to livestock ascends to 23.5% in this work, while 19.13% share human related molecular types. In parallel, the fact that most AMR isolates, as well as the *mecA*-encoding MRSA, belong to human-associated molecular types provide indication of human disturbance exerted upon wildlife, namely through additive feeds containing xenobiotics for metaphylactic purposes in livestock farms (e.g., aminoglycosides, polymyxins,

554 penicillin and tetracyclines) and also through the environmental contamination of soils and
555 watersheds. In fact, reinforcing the relevance of human disturbance upon ecological settings,
556 our modelling approaches clearly indicate that agricultural land cover and livestock production
557 (i.e. swine abundance) are linked to the occurrence of antimicrobial resistant *S. aureus*. The
558 AMR problem associated to extended use in animal husbandry is exacerbated, by poor
559 sanitation in rural settings and wastewater infrastructures that are inappropriate to deal with
560 antibiotic residues or resistant bacteria, contributing to significant environmental
561 contamination (Fouz et al., 2020). Manure application in agricultural soils leads to the
562 infiltration of xenobiotic and AMR bacteria into soil, then into groundwaters, while the direct
563 discharges of effluents into watersheds contaminate superficial waters (Silva et al., 2020).
564 These water bodies are subsequently used by wild animals and are exploited for raw water
565 uptake for human drinking purposes, potentially perpetuating the cycle of AMR
566 bacteria/xenobiotics in several spheres. Nevertheless, in our study, the presence of
567 antimicrobial resistant *S. aureus* was not related to the percentage of water bodies coverage
568 of the sampled municipalities, ~~which then suggests~~suggesting that other sources might be
569 more influential in the landscape context of the monitored areas in Portugal. Moreover,
570 antimicrobials naturally produced by environmental bacteria may also exert selective pressure.
571
572 **CONCLUSION**
573
574 Our results suggest that carriage of antimicrobial resistant *S. aureus* by wild ungulates may be
575 due to specific socio-ecological conditions linked to human-associated disturbance, like
576 proximity to livestock (namely, swine abundance) and landscape conversion towards
577 agricultural lands, a subject that deserves further evaluation in the future.

578 The results provided by this study highlight the relevance of maintaining a comprehensive
579 approach under the realm of One Health by dissecting the several components at the human,

environment and livestock interfaces that may act upon the lifecycle of antimicrobials and AMR bacteria. It has become clear that even though wildlife does not come in contact with antimicrobials directly, they have a role in the maintenance and dispersal of antimicrobial resistance determinants. The fact that some resistance determinants, like *mecA*, or the presence of linezolid-resistant isolates, that are ~~more~~ usually linked to human-associated strains are also present in wild boar and red deer, reveals that additional measures must be taken in order to prevent the indirect sources of contamination. There is an obvious need to mitigate routes of transmission of AMR bacteria and ARG to wildlife, with particular priority to epidemiological surveillance studies that integrate the environmental context to understand the complex dynamics of AMR and then, intervene. Prevention of further spread of AMR bacteria is of the utmost importance and will require a multidisciplinary approach involving all stakeholders.

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ETHICAL APPROVAL

Ethical review and approval were waived for this study since the samples analysed here were obtained in the scope of recreational hunting activities and donated for scientific purposes. No animals were sacrificed for the purpose of this study. None of the authors were responsible for the death of any animal. All applicable institutional and/or national/international guidelines for the use of animal specimens have been followed.

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REFERENCES

Arnold, T. W. (2010). Uninformative Parameters and Model Selection Using Akaike's Information Criterion. *The Journal of Wildlife Management, 74*(6), 1175-1178. doi: https://doi.org/10.1111/j.1937-2817.2010.tb01236.x

Barton, K. (2015). MuMIn: Multi-Model Inference. R package version 1.43.17.

Bates, D., Mächler, M., Bolker, B., & Walker, S. (2015). Fitting Linear Mixed-Effects Models Using lme4. *2015, 67*(1), 48 %J Journal of Statistical Software. doi: 10.18637/jss.v067.i01

Bencatel J., Sabino-Marques H., Álvares F., Moura A.E., & Barbosa A.M. (2019). *Atlas de Mamíferos de Portugal*. Universidade de Évora.

Bikandi, J., Millán, R. S., Rementeria, A., & Garaizar, J. (2004). In silico analysis of complete bacterial genomes: PCR, AFLP–PCR and endonuclease restriction. *Bioinformatics, 20*(5), 798-799. doi: 10.1093/bioinformatics/btg491 %J Bioinformatics

Carrasco-Garcia, R., Barasona, J. A., Gortazar, C., Montoro, V., Sanchez-Vizcaino, J. M., & Vicente, J. (2016). Wildlife and livestock use of extensive farm resources in South Central Spain: implications for disease transmission. *European Journal of Wildlife Research, 62*(1), 65-78. doi: 10.1007/s10344-015-0974-9

Diaz, R., Ramalheira, E., Afreixo, V., & Gago, B. (2016). Methicillin-resistant Staphylococcus aureus carrying the new mecC gene--a meta-analysis. *Diagn Microbiol Infect Dis, 84*(2), 135-140. doi: 10.1016/j.diagmicrobio.2015.10.014

Direção-Geral do Território - Ministério do Ambiente e Ação Climática - Portugal. (2018, Last Update). Carta de Uso e Ocupação do Solo para 2018.

Enright, M. C., Day, N. P., Davies, C. E., Peacock, S. J., & Spratt, B. G. (2000). Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of Staphylococcus aureus. *Journal of Clinical Microbiology, 38*(3), 1008-1015. doi: 10.1128/JCM.38.3.1008-1015.2000

EUCAST. (2021). Breakpoint tables for interpretation of MICs and zone diameters. https://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_11.0_Breakpoint_Tables.pdf

European Food Safety Authority. (2012). Technical specifications on the harmonised monitoring and reporting of antimicrobial resistance in methicillin-resistant Staphylococcus aureus in food-producing animals and food. *Efsa Journal*.

F. Dormann, C., M. McPherson, J., B. Araújo, M., Bivand, R., Bolliger, J., Carl, G., . . . Wilson, R. (2007). Methods to account for spatial autocorrelation in the analysis of species distributional data: a review. *Ecography, 30*(5), 609-628. doi: https://doi.org/10.1111/j.2007.0906-7590.05171.x

Feßler, A. T., Thomas, P., Mühldorfer, K., Grobbel, M., Brombach, J., Eichhorn, I., . . . Schwarz, S. (2018). Phenotypic and genotypic characteristics of Staphylococcus aureus isolates from zoo and wild animals. *Veterinary Microbiology, 218*, 98-103. doi: https://doi.org/10.1016/j.vetmic.2018.03.020

Fick, S. E., & Hijmans, R. J. (2017). WorldClim 2: new 1-km spatial resolution climate surfaces for global land areas. *37*(12), 4302-4315. doi: https://doi.org/10.1002/joc.5086

Fouz, N., Pangesti, K. N. A., Yasir, M., Al-Malki, A. L., Azhar, E. I., Hill-Cawthorne, G. A., & Abd El Ghany, M. (2020). The Contribution of Wastewater to the Transmission of Antimicrobial Resistance in the Environment: Implications of Mass Gathering Settings. *Tropical medicine and infectious disease, 5*(1), 33. doi: 10.3390/tropicalmed5010033

García-Álvarez, L., Holden, M. T., Lindsay, H., Webb, C. R., Brown, D. F., Curran, M. D., . . . Holmes, M. A. (2011). Meticillin-resistant Staphylococcus aureus with a novel mecA homologue in human and bovine populations in the UK and Denmark: a descriptive study. *Lancet Infect Dis, 11*(8), 595-603. doi: 10.1016/s1473-3099(11)70126-8

668 Gómez, P., Lozano, C., González-Barrio, D., Zarazaga, M., Ruiz-Fons, F., & Torres, C. (2015). High
669 prevalence of methicillin-resistant Staphylococcus aureus (MRSA) carrying the mecC
670 gene in a semi-extensive red deer (Cervus elaphus hispanicus) farm in Southern Spain.
671 *Vet Microbiol, 177*(3-4), 326-331. doi: 10.1016/j.vetmic.2015.03.029
672 Gonçalves, P., Alcobia, S., Simões, L., & Santos-Reis, M. (2012). Effects of management options
673 on mammal richness in a Mediterranean agro-silvo-pastoral system. *Agroforestry*
674 *Systems, 85*(3), 383-395. doi: 10.1007/s10457-011-9439-7
675 Heaton, C. J., Gerbig, G. R., Sensius, L. D., Patel, V., & Smith, T. C. (2020). Staphylococcus aureus
676 Epidemiology in Wildlife: A Systematic Review. *Antibiotics-Basel, 9*(2), 28. doi:
677 10.3390/antibiotics9020089
678 Hoekstra, J., Zomer, A. L., Rutten, V. P. M. G., Benedictus, L., Stegeman, A., Spaninks, M. P., . .
679 . Koop, G. (2020). Genomic analysis of European bovine Staphylococcus aureus from
680 clinical versus subclinical mastitis. *Scientific Reports, 10*(1), 18172. doi:
681 10.1038/s41598-020-75179-2
682 Hunter, P. R., & Gaston, M. A. (1988). Numerical index of the discriminatory ability of typing
683 systems: an application of Simpson's index of diversity. *J Clin Microbiol, 26*(11), 2465-
684 2466. doi: 10.1128/jcm.26.11.2465-2466.1988
685 Instituto Nacional de Estatística. (2019, Last Update). Efetivo animal (N.º) das explorações
686 agrícolas por Localização geográfica (NUTS - 2013) e Espécie animal.
687 Instituto Nacional de Estatística. (2020, Last Update). Densidade populacional (N.º/ km²) por
688 Local de residência (NUTS - 2013).
689 Johny, B. (2019). Characterization of Methicillin-resistant Staphylococcus aureus Isolated from
690 Nearby Hospitals from two Different Countries. *J Pure Appl Microbiol., 13*(3), 1683-
691 1688.
692 Kang, H. Y., Moon, D. C., Mechesso, A. F., Choi, J.-H., Kim, S.-J., Song, H.-J., . . . Lim, S.-K. (2020).
693 Emergence of cfr-Mediated Linezolid Resistance in Staphylococcus aureus Isolated
694 from Pig Carcasses. *9*(11), 769.
695 Kozajda, A., Jeżak, K., & Kapsa, A. (2019). Airborne Staphylococcus aureus in different
696 environments-a review. *Environmental science and pollution research international,*
697 *26*(34), 34741-34753. doi: 10.1007/s11356-019-06557-1
698 Laguna, E., Carpio, A. J., Vicente, J., Barasona, J. A., Triguero-Ocaña, R., Jiménez-Ruiz, S., . . .
699 Acevedo, P. (2021). The spatial ecology of red deer under different land use and
700 management scenarios: Protected areas, mixed farms and fenced hunting estates.
701 *Science of the Total Environment, 786*, 147124. doi:
702 https://doi.org/10.1016/j.scitotenv.2021.147124
703 Laxminarayan, R., Duse, A., Wattal, C., Zaidi, A. K., Wertheim, H. F., Sumpradit, N., . . . Cars, O.
704 (2013). Antibiotic resistance-the need for global solutions. *Lancet Infect Dis, 13*(12),
705 1057-1098. doi: 10.1016/s1473-3099(13)70318-9
706 Leão, C., Amaro, A., Albuquerque, T., & Clemente, L. (2021). *Whole Genome Sequencing of*
707 *Linezolid-Resistant Staphylococcus aureus ST398 From Healthy Pigs In Portugal*. Paper
708 presented at the OneHealth EJP 2021.
709 Letunic, I., & Bork, P. (2021). Interactive Tree Of Life (iTOL) v5: an online tool for phylogenetic
710 tree display and annotation. *Nucleic acids research, 49*(W1), W293-W296. doi:
711 10.1093/nar/gkab301 %J Nucleic Acids Research
712 Lin, J., Xu, P., Peng, Y., Lin, D., Ou, Q., Zhang, T., . . . Yao, Z. (2017). Prevalence and
713 characteristics of Staphylococcus aureus and methicillin-resistant Staphylococcus
714 aureus nasal colonization among a community-based diabetes population in Foshan,
715 China. *Journal of diabetes investigation, 8*(3), 383-391. doi: 10.1111/jdi.12591
716 Luzzago, C., Lauzi, S., Ehricht, R., Monecke, S., Trogu, T., Pedrotti, L., . . . Piccinini, R. (2019).
717 Staphylococcus aureus nasal and intestinal carriage by free-ranging red deer: evidence
718 of human, domestic and wild animal lineages. *International Journal of Infectious*
719 *Diseases, 79*, 21-22. doi: 10.1016/j.ijid.2018.11.067

720 Magiorakos, A. P., Srinivasan, A., Carey, R. B., Carmeli, Y., Falagas, M. E., Giske, C. G., . . .
721 Monnet, D. L. (2012). Multidrug-resistant, extensively drug-resistant and pandrug-
722 resistant bacteria: an international expert proposal for interim standard definitions for
723 acquired resistance. *Clinical Microbiology and Infection, 18*(3), 268-281. doi:
724 10.1111/j.1469-0691.2011.03570.x
725 Mairi, A., Touati, A., Pantel, A., Zenati, K., Martinez, A. Y., Dunyach-Remy, C., . . . Lavigne, J.-P.
726 (2019). Distribution of Toxinogenic Methicillin-Resistant and Methicillin-Susceptible
727 Staphylococcus aureus from Different Ecological Niches in Algeria. *11*(9), 500.
728 Manel, S., Williams, H. C., & Ormerod, S. J. (2001). Evaluating presence–absence models in
729 ecology: the need to account for prevalence. *Journal of Applied Ecology, 38*(5), 921-
730 931. doi: https://doi.org/10.1046/j.1365-2664.2001.00647.x
731 Marchesi, J. R., Sato, T., Weightman, A. J., Martin, T. A., Fry, J. C., Hiom, S. J., & Wade, W. G.
732 (1998). Design and Evaluation of Useful Bacterium-Specific PCR Primers That Amplify
733 Genes Coding for Bacterial 16S rRNA. *64*(2), 795-799. doi: doi:10.1128/AEM.64.2.795-
734 799.1998
735 Marshall, B. M., & Levy, S. B. (2011). Food animals and antimicrobials: impacts on human
736 health. *Clin Microbiol Rev, 24*(4), 718-733. doi: 10.1128/cmr.00002-11
737 Martineau, F., Picard, F. J., Roy, P. H., Ouellette, M., & Bergeron, M. G. (1998). Species-specific
738 and ubiquitous-DNA-based assays for rapid identification of Staphylococcus aureus. *J
739 Clin Microbiol, 36*(3), 618-623. doi: 10.1128/jcm.36.3.618-623.1998
740 Massei, G., Kindberg, J., Licoppe, A., Gačić, D., Šprem, N., Kamler, J., . . . Náhlik, A. (2015). Wild
741 boar populations up, numbers of hunters down? A review of trends and implications
742 for Europe. *Pest Manag Sci, 71*(4), 492-500. doi: 10.1002/ps.3965
743 Matrat, L., Plaisant, F., Barreto, C., Claris, O., & Butin, M. (2020). Increasing use of linezolid in
744 a tertiary NICU during a 10-year period: reasons and concerns for the future.
745 *Antimicrobial Resistance & Infection Control, 9*(1), 156. doi: 10.1186/s13756-020-
746 00818-2
747 Matuszewska, M., Murray, G. G. R., Harrison, E. M., Holmes, M. A., & Weinert, L. A. (2020). The
748 Evolutionary Genomics of Host Specificity in Staphylococcus aureus. *Trends in
749 Microbiology, 28*(6), 465-477. doi: 10.1016/j.tim.2019.12.007
750 Monecke, S., Gavier-Widén, D., Hotzel, H., Peters, M., Guenther, S., Lazaris, A., . . . Ehricht, R.
751 (2016). Diversity of Staphylococcus aureus Isolates in European Wildlife. *PLOS ONE,
752 11*(12), e0168433. doi: 10.1371/journal.pone.0168433
753 Monecke, S., Gavier-Widen, D., Mattsson, R., Rangstrup-Christensen, L., Lazaris, A., Coleman,
754 D. C., . . . Ehricht, R. (2013). Detection of mecC-Positive Staphylococcus aureus (CC130-
755 MRSA-XI) in Diseased European Hedgehogs (Erinaceus europaeus) in Sweden. *PLOS
756 ONE, 8*(6), e66166. doi: 10.1371/journal.pone.0066166
757 Nazareth, R., Gonçalves-Pereira, J., Tavares, A., Miragaia, M., de Lencastre, H., Silvestre, J., . . .
758 Póvoa, P. (2012). Infeção por staphylococcus aureus meticilina-resistente da
759 comunidade em Portugal. *Revista Portuguesa de Pneumologia, 18*(1), 34-38. doi:
760 https://doi.org/10.1016/j.rppneu.2011.05.007
761 Nübel, U., Roumagnac, P., Feldkamp, M., Song, J.-H., Ko, K. S., Huang, Y.-C., . . . Achtman, M.
762 (2008). Frequent emergence and limited geographic dispersal of methicillin-resistant
763 Staphylococcus aureus. *Proceedings of the National Academy of Sciences, 105*(37),
764 14130. doi: 10.1073/pnas.0804178105
765 Ochoa, S. A., Cruz-Córdova, A., Mancilla-Rojano, J., Escalona-Venegas, G., Esteban-Kenel, V.,
766 Franco-Hernández, I., . . . Xicohtencatl-Cortes, J. (2020). Control of Methicillin-
767 Resistant Staphylococcus aureus Strains Associated With a Hospital Outbreak Involving
768 Contamination From Anesthesia Equipment Using UV-C. *Frontiers in Microbiology, 11*,
769 600093-600093. doi: 10.3389/fmicb.2020.600093
770 Palmeira, J. D., Cunha, M. V., Carvalho, J., Ferreira, H., Fonseca, C., & Torres, R. T. (2021).
771 Emergence and Spread of Cephalosporinases in Wildlife: A Review. *11*(6), 1765.

Field Code Changed

772 Palomo, L., Gisbert, J., & Blanco, J. (2007). Atlas Y Libro Rojo De Los Mamíferos Terrestres De
773 España.
774 Paradis, E., & Schliep, K. (2018). ape 5.0: an environment for modern phylogenetics and
775 evolutionary analyses in R. *Bioinformatics, 35*(3), 526-528. doi:
776 10.1093/bioinformatics/bty633 %J Bioinformatics
777 Peres, D., Pina, E., & Fonseca Cardoso, M. (2011). Methicillin-Resistant Staphylococcus Aureus
778 (MRSA) in a Portuguese hospital and its risk perception by health care professionals.
779 *Revista Portuguesa de Saúde Pública, 29*(2), 132-139. doi:
780 https://doi.org/10.1016/S0870-9025(11)70017-7
781 Plaza-Rodríguez, C., Alt, K., Grobbel, M., Hammerl, J. A., Irrgang, A., Szabo, I., . . . Tenhagen, B.-
782 A. (2021). Wildlife as Sentinels of Antimicrobial Resistance in Germany? *, 7*(1251). doi:
783 10.3389/fvets.2020.627821
784 Porrero, M. C., Mentaberre, G., Sánchez, S., Fernández-Llario, P., Casas-Díaz, E., Mateos, A., . .
785 . Domínguez, L. (2014). Carriage of Staphylococcus aureus by free-living wild animals
786 in Spain. *Appl Environ Microbiol, 80*(16), 4865-4870. doi: 10.1128/aem.00647-14
787 Porrero, M. C., Valverde, A., Fernández-Llario, P., Díez-Guerrier, A., Mateos, A., Lavín, S., . . .
788 Domínguez, L. (2014). Staphylococcus aureus carrying mecC gene in animals and urban
789 wastewater, Spain. *Emerg Infec Dis, 20*(5), 899-901. doi: 10.3201/eid2005.130426
790 Price, L. B., Stegger, M., Hasman, H., Aziz, M., Larsen, J., Andersen, P. S., . . . Aarestrup, F. M.
791 (2012). Staphylococcus aureus CC398: Host Adaptation and Emergence of Methicillin
792 Resistance in Livestock. *mBio, 3*(1), e00305-00311. doi: 10.1128/mBio.00305-11
793 Resch, G., François, P., Morisset, D., Stojanov, M., Bonetti, E. J., Schrenzel, J., . . . Moreillon, P.
794 (2013). Human-to-Bovine Jump of Staphylococcus aureus CC8 Is Associated with the
795 Loss of a β-Hemolysin Converting Prophage and the Acquisition of a New
796 Staphylococcal Cassette Chromosome. *PLOS ONE, 8*(3), e58187. doi:
797 10.1371/journal.pone.0058187
798 Richardson, E. J., Bacigalupe, R., Harrison, E. M., Weinert, L. A., Lycett, S., Vrieling, M., . . .
799 Fitzgerald, J. R. (2018). Gene exchange drives the ecological success of a multi-host
800 bacterial pathogen. *Nature Ecology & Evolution, 2*(9), 1468-1478. doi:
801 10.1038/s41559-018-0617-0
802 Robin, X., Turck, N., Hainard, A., Tiberti, N., Lisacek, F., Sanchez, J.-C., & Müller, M. (2011).
803 pROC: an open-source package for R and S+ to analyze and compare ROC curves. *BMC*
804 *Bioinformatics, 12*(1), 77. doi: 10.1186/1471-2105-12-77
805 Ruffing, U., Alabi, A., Kazimoto, T., Vubil, D. C., Akulenko, R., Abdulla, S., . . . Herrmann, M.
806 (2017). Community-Associated Staphylococcus aureus from Sub-Saharan Africa and
807 Germany: A Cross-Sectional Geographic Correlation Study. *Scientific Reports, 7*(1), 154.
808 doi: 10.1038/s41598-017-00214-8
809 Ruiz-Ripa, L., Alcalá, L., Simón, C., Gómez, P., Mama, O. M., Rezusta, A., . . . Torres, C. (2019).
810 Diversity of Staphylococcus aureus clones in wild mammals in Aragon, Spain, with
811 detection of MRSA ST130-mecC in wild rabbits. *Journal of Applied Microbiology,*
812 *127*(1), 284-291. doi: https://doi.org/10.1111/jam.14301
813 Salgueiro, V., Manageiro, V., Bandarra, N. M., Ferreira, E., Clemente, L., & Caniça, M. (2020).
814 Genetic Relatedness and Diversity of Staphylococcus aureus from Different Reservoirs:
815 Humans and Animals of Livestock, Poultry, Zoo, and Aquaculture. *8*(9), 1345.
816 Schaumburg, F., Alabi, A. S., Köck, R., Mellmann, A., Kremsner, P. G., Boesch, C., . . . Peters, G.
817 (2012). Highly divergent Staphylococcus aureus isolates from African non-human
818 primates. *Environmental Microbiology Reports, 4*(1), 141-146. doi:
819 https://doi.org/10.1111/j.1758-2229.2011.00316.x
820 Seinige, D., Von Altrock, A., & Kehrenberg, C. (2017). Genetic diversity and antibiotic
821 susceptibility of Staphylococcus aureus isolates from wild boars. *Comp Immunol*
822 *Microbiol Infect Dis, 54*, 7-12. doi: 10.1016/j.cimid.2017.07.003

Field Code Changed

Silva, V., Caniça, M., Capelo, J. L., Igrejas, G., & Poeta, P. (2020). Diversity and genetic lineages of environmental staphylococci: a surface water overview. *Fems Microbiology Ecology, 96*(12). doi: 10.1093/femsec/fiaa191

Silva, V., Gabriel, S. I., Borrego, S. B., Tejedor-Junco, M. T., Manageiro, V., Ferreira, E., . . . Poeta, P. (2021). Antimicrobial Resistance and Genetic Lineages of Staphylococcus aureus from Wild Rodents: First Report of mecC-Positive Methicillin-Resistant S. aureus (MRSA) in Portugal. *11*(6), 1537.

Silva, V., Oliveira, A., Manageiro, V., Caniça, M., Contente, D., Capita, R., . . . Poeta, P. (2021). Clonal Diversity and Antimicrobial Resistance of Methicillin-Resistant Staphylococcus pseudintermedius Isolated from Canine Pyoderma. *9*(3), 482.

Sousa, M., Silva, N., Manageiro, V., Ramos, S., Coelho, A., Gonçalves, D., . . . Poeta, P. (2017). First report on MRSA CC398 recovered from wild boars in the north of Portugal. Are we facing a problem? *Science of the Total Environment, 596-597*, 26-31. doi: https://doi.org/10.1016/j.scitotenv.2017.04.054

Sousa, M., Silva, V., Silva, A., Silva, N., Ribeiro, J., Tejedor-Junco, M. T., . . . Poeta, P. (2020). Staphylococci among Wild European Rabbits from the Azores: A Potential Zoonotic Issue? *Journal of Food Protection, 83*(7), 1110-1114. doi: 10.4315/0362-028x.jfp-19-423

Stegger, M., Andersen, P. S., Kearns, A., Pichon, B., Holmes, M. A., Edwards, G., . . . Larsen, A. R. (2012). Rapid detection, differentiation and typing of methicillin-resistant Staphylococcus aureus harbouring either mecA or the new mecA homologue mecA(LGA251). *Clin Microbiol Infect, 18*(4), 395-400. doi: 10.1111/j.1469-0691.2011.03715.x

Strube, M. L., Hansen, J. E., Rasmussen, S., & Pedersen, K. (2018). A detailed investigation of the porcine skin and nose microbiome using universal and Staphylococcus specific primers. *Scientific Reports, 8*(1), 12751. doi: 10.1038/s41598-018-30689-y

Torres, R. T., Carvalho, J., Cunha, M. V., & Fonseca, C. (2019). Antimicrobial Resistance and Ecology: A Dialog Yet to Begin. *Ecohealth, 16*(3), 402-403. doi: 10.1007/s10393-019-01438-y

Torres, R. T., Carvalho, J., Cunha, M. V., Serrano, E., Palmeira, J. D., & Fonseca, C. (2020). Temporal and geographical research trends of antimicrobial resistance in wildlife - A bibliometric analysis. *One Health, 11*, 100198. doi: https://doi.org/10.1016/j.onehlt.2020.100198

Torres, R. T., Fernandes, J., Carvalho, J., Cunha, M. V., Caetano, T., Mendo, S., . . . Fonseca, C. (2020). Wild boar as a reservoir of antimicrobial resistance. *Science of the Total Environment, 717*, 135001. doi: https://doi.org/10.1016/j.scitotenv.2019.135001

Vancraeynest, D., Haesebrouck, F., Deplano, A., Denis, O., Godard, C., Wildemauwe, C., & Hermans, K. (2006). International dissemination of a high virulence rabbit Staphylococcus aureus clone. *J Vet Med B Infect Dis Vet Public Health, 53*(9), 418-422. doi: 10.1111/j.1439-0450.2006.00977.x

Vittecoq, M., Godreuil, S., Prugnolle, F., Durand, P., Brazier, L., Renaud, N., . . . Renaud, F. (2016). Antimicrobial resistance in wildlife. *53*(2), 519-529. doi: https://doi.org/10.1111/1365-2664.12596

Zuur, A. F., Hilbe, J. M., & Ieno, E. N. (2013). *A beginner's guide to GLM and GLMM with R.*: Newburgh: Highland Statistics Ltd.

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873 **Table 1 – Modelling hypothesis, rationale, prediction, and variable description.** All variables
874 were represented according to municipality administrative regions.

Hypothesis	Rationale	Prediction	Variables	Variables' code
H1 Land Use	The differential profile of land use along municipalities may influence the overall AMR occurrence and dispersion, as different species habit particular types of land cover	We expect higher percentage of agricultural areas and water bodies to influence positively the AMR occurrence	Agriculture coverage (%) Artificial areas coverage (%) Forest coverage (%) Water bodies coverage (%)	*Agri_cov* *Artif_cov* *Forest_cov* *Water_cov*
H2 Livestock abundance	The sympatry between livestock and wildlife animals is known to promote disease transmission	We expect higher livestock abundance to promote the AMR occurrence	Cattle abundance (no. animals) Swine (no. animals) Poultry (no. animals)	*Cattle_abun* *Swi_abun* *Poul_abun*
H3 Anthropogenic disturbance	The anthropogenic influence is known to be a factor that promotes the transmission of AMR bacteria and AMR determinants to the animal compartment	We expect that higher human density will increase AMR occurrence	Population density (no. people/km^2)	*Pop_dens*
H4 Wild ungulates presence	The interspecies transmission of AMR bacteria and AMR determinants is known to promote the maintenance within the animal compartment	We expect that the presence of different wild species will increase the AMR occurrence	Mufflon presence (binary) Roe deer presence (binary) Red deer presence (binary)	*Muff_pre* *Roe_pre* *Red_pre*
H5	The life history traits of the host,	We expect that a	Host species (Wild boar, red	*Host_spe*

Life-history traits	namely its species, sex or age can be associated with the AMR occurrence	particular species, sex, or age class is more prone to promote AMR occurrence	deer, fallow deer) Sex (Male, Female) Age (Adult, Juvenile)	 *Sex* *Age*
H6 Climate	The climate, namely the volume of precipitation, can influence animal feeding behaviour	We expect that driest zones will increase the AMR occurrence	Annual precipitation (mm) Driest month precipitation (mm) [Data were calculated by interpolations of observed data, representing average values for the period 1970–2000]	*Annual_prec* *Driest_prec*

875

876 **Table 2 - *Staphylococcus aureus* detection and molecular characterization.**

Animal species	No. tested animals	No. positive animals	Prevalence	MLST[a] (No. of isolates)	MLST[b] (No. of isolates)	*spa* type (No. of isolates)
Fallow deer	23	5	21.7% (95% CI: 3.5-40%)	ST1 (1)	ST1 (6)	t127 (6)
				ST5(1)	ST5 (2)	t002 (2)
				ST133 (1)	ST133 (4)	t3583 (4)
Red deer	54	29	53.7% (95% CI: 40-67.4%)	t9632/ST1 (1) t1533/ST1 (1) t2207/ST1 (1)	ST1 (13)	t9632 (7), t1533 (5), t2207 (1)
					ST133 (3)	t3583 (3)
				ST425 (1)	ST425 (16)	t6386 (16)
					ST544 (3)	t7386 (3)
				t3750/ST2328 (1)	ST2328 (4)	t3750 (4)
				t9304/ST2678 (1) t11502/ST2678 (1)	ST2678 (49)	t11502 (45), t9304 (4)
						t1793 (1)
						t701 (2)
						t10205 (1)

Wild boar	177	57	32.2% (95% CI: 25.3-39.2%)	t1407/ST1 (1)	ST1 (6)	t1407 (4), t1533 (2)
					ST5 (15)	t002 (15)
				ST8(1)	ST72 (8)	t148 (8)
				t1181/ ST133 (1)	ST133 (4)	t3583 (3), t1181 (1)
				t011/ST398 (1)	ST398 (17)	t011 (9), t034 (8)
				t11232/ST425 (1)	ST425 (19)	t11232 (15)
				ST544 (2)	ST544 (15)	t7386 (3*, 12)
				ST772 (1)	ST772 (2)	t098 (2)
				t11230/ST2328 (1)	ST2328 (20)	t3750 (18), t11230 (2)
					ST2678 (63)	t11502 (37), t12939 (26)
						t1773 (1)
						t4279 (2)
						t11225 (2)

[a]Experimentally determined MLST (20 isolates); [b]Inferred sequence type based on presumable *Spa* type/ST associations (comprises all isolates, including [a]); * represents MRSA isolates.

Table 3 – Resistance profile of multidrug resistant *Staphylococcus aureus* isolates. GEN – Gentamiycin; LIN – Linezolid; TET – Tetracycline; CXI – Cefoxitin; CIP – Ciprofloxacin; BEN – Benzylpenicillin; OXA – Oxacillin; ERY – Erythromycin.

Host	Isolate ID	Resistance phenotype	*mecA*	*mecC*
Wild boar	0645	GEN-LIN-TET	-	-
Wild boar	0649	CXI-CIP-GEN-BEN-OXA	-	-
Wild boar	0650	CXI-CIP-GEN-LIN-BEN-OXA	-	-
Wild boar	0651	CXI-CIP-GEN-BEN-OXA	-	-
Wild boar	0698	CXI-CIP-GEN-BEN	-	-
Wild boar	0959	CXI-BEN-OXA	+	-
Wild boar	0960	CXI-BEN-OXA	+	-
Wild boar	0980	CXI-ERY-GEN-BEN	-	-
Wild boar	1012	CXI-GEN-TET-BEN-OXA	-	-
Wild boar	1045	CXI-ERY-BEN	+	-

Table 4 – Best models for each of the working hypothesis selected according to the model's AICc and ΔAICc.

Models	df	AICc	ΔAICc	AICc weight
H1 – Land Use hypothesis				
Agriculture + (1|Municipality)	3	259.0	0.00	0.549
Agriculture + Water bodies + (1|Municipality)	4	260.6	1.60	0.246
Agriculture + Artificial + (1|Municipality)	4	261.0	1.98	0.205
H2 – Livestock abundance hypothesis				
Swine + (1|Municipality)	3	263.0	0.00	0.560
Swine + Poultry + (1|Municipality)	4	264.7	1.71	0.238
H3 – Anthropogenic disturbance hypothesis				
Population density + (1|Municipality)	3	264.6	0.00	1.000
H4 – Wild ungulates presence hypothesis				
Mufflon + (1|Municipality)	3	259.9	0.00	0.721
Mufflon + Roe deer + (1|Municipality)	4	261.8	1.89	0.279
H5 – Life-history traits hypothesis				
Sex + (1|Municipality)	3	262.72	0.00	0.48
Sex + Age + (1|Municipality)	4	263.84	1.13	0.27
Sex + Host + (1|Municipality)	5	264.0	1.28	0.25
H6 – Climate hypothesis				
Annual precipitation + (1|Municipality)	3	261.5	0.00	1.000
H7 – Multiple variable hypothesis				
Agriculture + Sex + Swine + (1|Municipality)	**5**	**252.9**	**0.00**	**0.345**
Sex + Swine + Mufflon + (1|Municipality)	**5**	**253.2**	**0.31**	**0.295**
Agriculture + Sex + Swine + Mufflon + (1|Municipality)	**6**	**253.8**	**0.88**	**0.222**
Agriculture + Sex + (1|Municipality)	**4**	**254.8**	**1.82**	**0.138**

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Figure Captions

Figure 1 - Minimum spanning tree of *spa* types of *Staphylococcus aureus* isolates from wildlife species. Illustration of the evolutionary link between *S. aureus* isolates based on combined character data of *spa* typing. Node size is proportional to the number of isolates belonging to each *spa* type. Node colours correspond to host species: wild boar – green; red deer – purple; and fallow deer – orange. Shading represents clonal complexes with human-associated CCs coloured in reds and livestock-associated CCs coloured in blues.

Figure 2 – *Spa* type distribution across mainland Portugal districts. Pie charts represent the proportion of each *spa* type in the pointed district. Top 15 most frequent *spa* types countrywide are represented in colours. Districts represented in the map host ten or more isolates. Map was generated with QGIS software v3.16.

Figure 3 – Molecular diversity and antibiotic resistance profiling of *Staphylococcus aureus* isolates from wildlife species.

Figure 4 – Receiver Operating Characteristic Curve (ROC) for the average model of the best hypothesis. Area Under the Curve (AUC) = 0.8601

Supplementary Tables

Supplementary Table 1 - Complete characterization of the 303 *S. aureus* isolates by Sampling site, Host, Host's sex and age class, Sequence type, *Spa* type, Resistance phenotype and *mecA* and *mecC* amplification results.

Supplementary Table 2 – Principal Components Analysis with individual and cumulative proportions of variance and explanatory variables.

Supplementary Table 3 – *p*-values of Mann-Whitney non-parametric test (α=0.05) for hypotheses testing of explanatory variables derived from Principal Components Analysis.

Supplementary Table 4 - Confidence intervals (95%) of the average model for the best hypothesis.

Supplementary Figures