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Exploring the Antibiotic-Resistance Profile of *Staphylococcus aureus* Recovered from Portuguese Fermented Meat Products

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I dedicate this work to my beloved parents, whose unwavering love, sacrifices, and strength have been the foundation of all my achievements. To my siblings, for their constant encouragement and faith in me. To my sister Bochra, the greatest inspiration in my life, and to my little niece Miriam, whose joy and laughter bring light to my days. To my life partner, Farouk, whose love and presence have been my anchor through every challenge. And finally, to the little me who never stopped dreaming and working hard to turn those dreams into reality.

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LIST OF ABBREVIATIONS

- **aw** water activity
- **g** gram
- **L** litre
- **°C** Celsius
- **ml** millilitre
- **µl** microlitre
- **nm** nanometre
- **mm** millimetre

LIST OF ACRONYMS

- **AMR** Antimicrobial resistance
- **ARGs** Antibiotic resistance genes
- **ATU** Area of Technical Uncertainty
- **CDC** Centre for Disease Control and Prevention
- **CFU** Colony-forming units
- **CNC** Coagulase-negative cocci
- **Dim** Dimension
- **EFSA** European Food Safety Authority
- **EU** European Union
- **EUCAST** European Committee on Antimicrobial Susceptibility Testing
- **HGT** Horizontal gene transfer
- **LAB** Lactic acid bacteria
- **LPS** Lipopolysaccharide
- **MDR** Multidrug resistance
- **MRSA** Methicillin-resistant *S. aureus*
- **PBP** Penicillin-binding protein
- **PCA** Principal Component Analysis
- **PGI** Protected Geographical Indication
- **PVL** Panton-Valentine leucocidin
- **QC** Quality control
- **RTE** Ready-to-eat
- **S, R** Susceptibility / Resistance
- **SFP** Staphylococcal food poisoning
- **TSST** Toxic shock syndrome toxin
- **WGS** Whole Genome Sequencing

ABSTRACT

Chouriça and *alheira* are traditional Portuguese sausages with culinary, cultural, and economic significance. However, its artisanal production is often linked to non-standardised manufacturing processes and hygiene practices, which may result in contamination by foodborne pathogens and antimicrobial resistance (AMR) dissemination.

In this study, forty-seven *Staphylococcus aureus* isolates from *chouriça* (RTE, n=22) and *alheira* (non-RTE, n=25) sausages were subjected to antimicrobial susceptibility testing by disk diffusion following EUCAST guidelines, against 14 antibiotics. The resulting data were analysed performing descriptive analysis and Principal Component Analysis (PCA) with the AMR R Package.

Resistance was observed only to eight antibiotics, with 68.08% of isolates resistant to at least one agent and low rate of multidrug-resistant isolates (6.4%). All strains were susceptible to cefoxitin, indicating the absence of methicillin-resistant *S. aureu*s isolates. Benzylpenicillin resistance was most common, especially in RTE (50%) and non-RTE (40%), erythromycin resistance was higher in RTE (40.9%), and norfloxacin resistance appeared only in non-RTE products (16%). PCA extracted two components that explain the majority of resistance variation and revealed clear clustering by sausage type, indicating distinct resistance profiles between RTE and non-RTE sources.

Detection of antibiotic-resistant *S. aureus* in fermented sausages, particularly to antibiotics so common in human treatment, probably results from poor hygiene practices during manufacturing and improper fermentation, allowing the survival of human-derived strains in the end product. Improving hygiene is important to curb AMR spread; therefore future research should focus on resistance transmission and genotypic characterization.

Keywords: fermented sausages; foodborne pathogens; *Staphylococcus aureus*; antimicrobial resistance; EUCAST; Principal component analysis.

RESUMO

A chouriça e a alheira são enchidos tradicionais portugueses com importância culinária, cultural e económica. No entanto, a sua produção artesanal pode estar associada a processos de fabrico e práticas de higiene não padronizados, resultando na contaminação por agentes patogénicos de origem alimentar e na disseminação da resistência antimicrobiana (RAM).

Neste estudo, quarenta e sete isolados de *Staphylococcus aureus* provenientes de chouriça (RTE, n=22) e alheira (não RTE, n=25) foram submetidos a testes de suscetibilidade antimicrobiana por difusão em disco, seguindo as diretrizes da EUCAST, contra 14 antibióticos. Os dados resultantes foram analisados através de uma análise descritiva e de uma Análise de Componentes Principais (PCA) com o pacote AMR R.

Foi observada resistência apenas a oito antibióticos, com 68,1% dos isolados resistentes a pelo menos um agente e baixa taxa de isolados multirresistentes (6,4%). Todas as estirpes foram suscetíveis à cefoxitina, indicando a ausência de isolados de *S. aureus* resistentes à meticilina. A resistência à benzilpenicilina foi a mais comum, especialmente em RTE (50%) e não RTE (40%); a resistência à eritromicina foi maior em RTE (40,9%); e a resistência à norfloxacina apareceu apenas em isolados de não RTE (16%). A PCA mostrou dois componentes que explicavam a maior parte da variação da resistência e revelou um agrupamento claro por tipo de enchido, indicando perfis de resistência distintos entre isolados RTE e não RTE.

A deteção de *S. aureus* resistente a antibióticos em enchidos fermentados, particularmente aos antibióticos tão comuns no tratamento humano, resulta provavelmente de práticas de higiene inadequadas durante o fabrico e/ou fermentação inadequada, permitindo a sobrevivência de estirpes derivadas do ser humano no produto final. Melhorar a higiene é importante para conter a propagação da RAM, e futuras pesquisas devem concentrar-se na transmissão da resistência e na caracterização genotípica.

Palavras-chaves: enchidos fermentados; patógenos transmitidos por alimentos; *Staphylococcus aureus*; resistência antimicrobiana; EUCAST; análise de componentes principais.

I. INTRODUCTION

Foodborne pathogens pose a major threat to health worldwide. They are responsible for a variety of illnesses ranging from mild gastrointestinal disorders to serious, life-threatening conditions, and often lead to large-scale epidemics. The challenge is further intensified by the expanding presence and resilience of antimicrobial resistance (AMR) within these pathogens, making it more difficult to effectively manage and treat infections (WHO, 2022). AMR greatly undermines the efficacy of standard antibiotic treatments, leading to more severe illnesses and higher mortality rates (Almansour et al., 2023).

The World Health Organization (WHO) reports that food-borne diseases cause around 600 million illnesses and 420000 deaths worldwide each year, with children accounting for 30% of cases despite making up only 9% of the population (WHO, 2022). In addition, AMR-related infections are currently responsible for around 700000 deaths a year, a figure that could rise to 10 million a year by 2050 if left unattended (WHO, 2019).

In Portugal, fermented meat products are an integral part of the daily food consumption in the rural regions, and have also gained popularity in urban areas, where demand is growing rapidly. Traditionally fermented sausages are generally perceived as safe by consumers, due in part to the combined reduction in water activity (aw) and pH levels during processing, which effectively limits the growth of pathogenic bacteria along the manufacturing stages. Since raw meat is often contaminated, there is a risk that some pathogenic organisms will overcome the antimicrobial barriers applied during processing. Consequently, these pathogens can remain in the final product, posing significant problems for producers and public health officials. This issue has been investigated extensively by various research groups to enhance the safety of fermented meat products (Ananou et al., 2005; Ferreira et al., 2007a; Samelis and Metaxopoulos, 1999; Thévenot et al., 2005).

The expansion of the meat industry has led to an increase in the prevalence of meat-borne diseases, which can be caused by chemical hazards, zoonotic infections or environmental contaminants (Nkosi et al., 2020). Among these diseases, pathogenic bacteria are the most relevant, as they cause infections through zoonotic transmission or contamination during meat production and processing (Wang et al., 2021). The principal zoonotic bacterial pathogens associated with food-borne diseases and deaths globally are

Staphylococcus aureus, *Salmonella* spp., *Campylobacter* spp., *Listeria monocytogenes* (*L. monocytogenes*), *Escherichia coli*, *Shigella* spp., *Bacillus cereus*, *Yersinia enterocolitica*, *Clostridium botulinum*, *C. perfringens*, and *Vibrio cholera* (Abebe et al., 2020; Cremonesi et al., 2014).

Salmonellosis is the second most common food-borne gastrointestinal infection notified in the European Union in 2022, with 65 208 confirmed cases, becoming a leading cause of foodborne outbreaks in both EU Member States and non-EU countries. Listeriosis was the fifth most frequently reported zoonosis, with an EU notification rate of 0.62 cases per 100 000 population, representing an increase of 15.9% from 2021 and the highest rate since 2007. Despite this increase, the overall trend for listeriosis remained stable between 2018 and 2022. Listeriosis had a high case fatality rate of 18.1%, which was higher than the rates in 2021 and 2020 (EFSA, 2023)

In fermented food chains, microorganisms can harbour and transfer AMR genes to opportunistic or human pathogenic bacteria through horizontal gene transfer (Marshall and Levy, 2011). The rapidly increasing prevalence of antimicrobial-resistant pathogens is a serious public health problem worldwide (Gaze et al., 2013); and has enormous economic and commercial consequences (George, 2019). As a result, the European Food Safety Authority (EFSA) has recommended the assessment of antimicrobial resistance in starter cultures, with particular attention to ensuring the absence of transferable antimicrobial resistance factors (Sánchez Mainar et al., 2017; Talon and Leroy, 2011).

In addition, Multidrug Resistance (MDR) has been discovered in a variety of bacterial isolates from animal farms and food products in Portugal. For example, in 2013, fifty-five percent of *Salmonella* isolates from pigs and 61% from pork products were MDR. In 2016, fifty-six percent of *E. coli* isolates from poultry carcasses showed MDR, while in 2016 and 2017, *Enterobacteriaceae* from animal dung on poultry (71%) and pig (79%) farms showed MDR. These bacteria were resistant to tetracyclines, sulphonamides, chloramphenicol, amoxicillin, and trimethoprim (Amador et al., 2019; Clemente et al., 2013).

Antibiotic resistance limits the range of therapeutic options available and increases the risk of therapeutic failure and adverse clinical outcomes. However, no comprehensive research has yet been undertaken in Portugal to investigate the antimicrobial resistance profiles of common foodborne pathogens present in the traditional dry-fermented sausages produced in the North of the country. Given the global relevance of AMR, this study will focus on *S. aureus* strains isolated from these traditional sausages; and will evaluate their resistance profiles in the form of antibiograms. The results are expected to inform food safety authorities and to contribute to shape up future strategies to control and manage AMR pathogens in the food chain.

II. OBJECTIVES

This study aims to assess the prevalence and characterize the phenotypic profile of antibiotic-resistance of *Staphylococcus aureus* strains isolated from artisanal Portuguese ready-to-eat (RTE) and non-RTE sausages. This work will enable awareness, as well as an assessment of the containment and prevention measures to be implemented to manage multi-resistant food-borne bacteria throughout the food chain.

III. LITERATURE REVIEW

3.1. Portuguese fermented sausages

3.1.1 Traditional sausages in Portugal

Fermented raw sausages are traditionally characterized as products made from meat and fat, cut and minced, with or without the inclusion of offal, combined with seasonings, spices and permitted additives. These products undergo a controlled maceration or curing process and, in some cases, are smoked. In Portugal, the curing process is generally combined with dry-smoking, which imparts flavours and facilitates preservation (Santos et al., 2011). The diversity of fermented sausages is influenced by regional traditions, environmental factors and family recipes, creating a wide variety of products mainly in the northern region of Trás-os-Montes (districts of Vila Real and Bragança) and in the southern region of Alentejo (districts of Évora, Beja and Portalegre) (Fig. 1). Despite this diversity, their production is universally based on the processes of fermentation, dehydration and/or smoking, and subsequent maturation (Elías and Carrascosa, 2010; Ferreira and Mendes, 2010).

In Mediterranean countries, such as Portugal, the most common sausages are dried and spiced, with longer maturation periods than in Central and Northern Europe. In these regions, fermentation is often combined with smoking or a gentler curing process (Demeyer, 2014). In Portugal, most of the manufacturers that produce artisanal sausages do not inoculate any commercial starter or sugar, and so the fermentation process occurs naturally, driven by the indigenous microbiota, and progresses at a moderate rate, though room temperature conditions can make it challenging to standardize (Elias et al., 2014; Roseiro et al., 2010). This makes these sausages quite unique compared to the United States varieties, which are fermented more quickly at higher temperatures and dried for shorter periods, giving them distinct organoleptic characteristics (Elias et al., 2006; Ordöñez and de la Hoz, 2007; Talon et al., 2007).

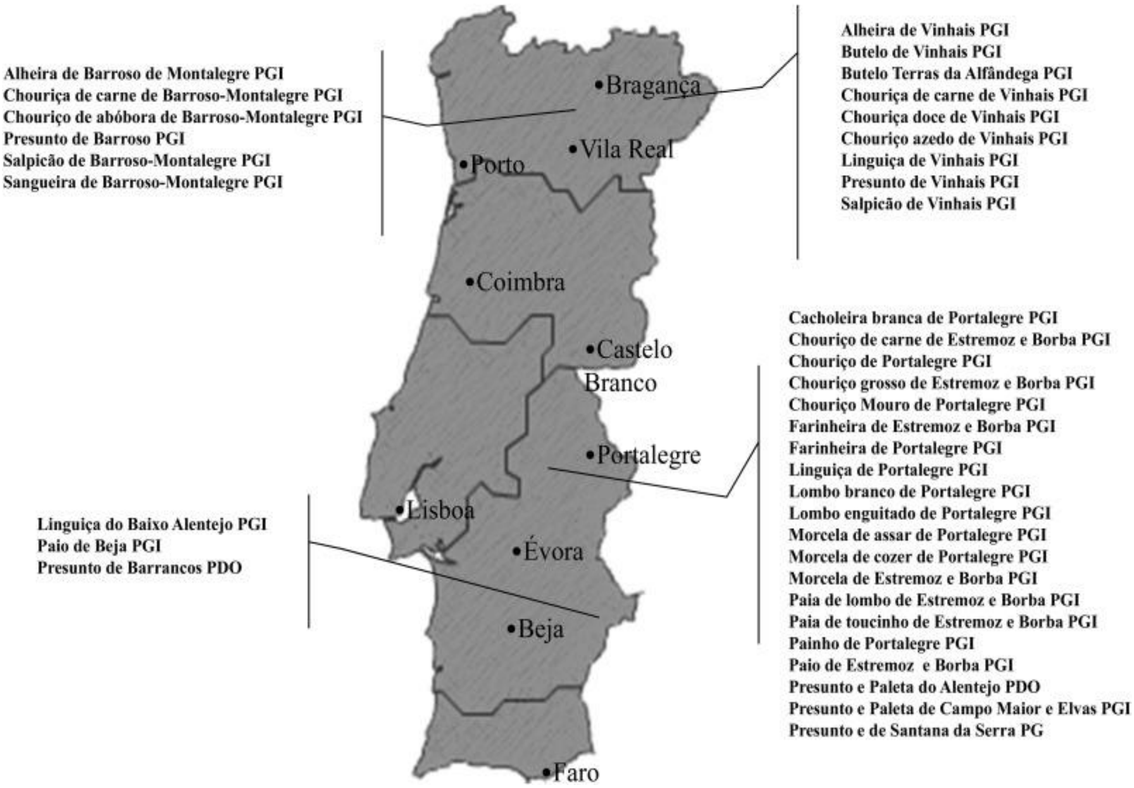


Figure 1: Classified traditional meat products from Portugal according to their geographical area of production

(Source: Marcos et al., 2016).

3.1.2 Types of sausages

Considering the great diversity of traditional Portuguese sausages, only the most important varieties will be described, focusing on their main distinctive characteristics (Albano et al., 2009; Couto, 2003):

1- *Alheira*: is a non-ready-to-eat (non-RTE) traditional smoked sausage from the region of Trás-os-Montes, Portugal, where it is recognized today as a product with Protected Geographical Indication (PGI) status. Originally created by Portuguese Jews to replace pork during the Inquisition, it is made from a mixture of meats (veal, pork, poultry or game), combined with bread, fat, spices, garlic and olive oil or lard.

2- *Chouriça*: is among the most popular Portuguese RTE sausages, known for its versatility and deep roots in traditional cuisine. There are various types of chouriça: meat-based, blood-based and honey-infused. The most widespread, chouriça de carne, is made from a mixture of lean and fatty pork, seasoned with chili paste, garlic, salt and other spices or additives. It is classified as traditional (with natural casings), *corrente* (with boiled or cured rind) or *extra* (with selected lean cuts).

3- *Linguiça*: is a smoked sausage made exclusively from minced pork meat and fat, seasoned with spices and optionally additives, fat free content and < 2.5 times the total protein content.

4- *Salpicão*: is a traditional sausage with different preparation methods and seasonings. It consists of a mixture of pork loin or other lean cuts, combined with firm fat. The large pieces of loin are marinated in a brine of wine, salt, pepper and garlic before being smoked. The length of the smoking process varies according to the region of production.

3.1.3 Production process

The production of sausages involves several key steps, starting with meat and fat selection that involves choosing the quality raw materials for the various types of sausage, while removing parts that are unsuitable. The proportion of muscle to fat in meat is a key factor, since fat levels considerably impact the sensory characteristics of cured products, in particular their texture, succulence and taste. In addition, fat helps to retain moisture in the muscle fibres, which accelerates fermentation during the curing stage. When selecting meat, it is important to ensure a balanced composition of lean meat and firm fat (Marcos et al., 2016).

Meat chopping involves cutting the raw materials (meat and fat) into appropriate-sized pieces, which can be done either manually or mechanically. Manual cutting is widely used in the preparation of artisanal sausages. The degree of size reduction affects water removal, with larger pieces retaining moisture for longer, while smaller pieces allow for more effective binding and faster moisture loss. During the preparation of the batter, extra ingredients are incorporated to the main raw materials and left to macerate.

The maturation phase involves the absorption of salt and condiments by the meat, followed by a loss of water and myofibrillar proteins. This stage also enhances microbial activity, leading to the generation of metabolic by-products. For the filling process, the matured mix is filled into casings, which help to shape and protect the mass from external influences, including microbial contamination. These casings must not introduce any contaminants. In Portugal, drying, often associated with smoking, continues the physical, chemical, biochemical and microbiological processes initiated earlier during maceration, resulting in a product with organoleptic and preservation qualities distinct from those of the raw material (Elias et al., 2006).

3.2. Microbiology of dry fermented meat sausages

The natural microbiota presents in raw materials and the environment in which they are processed play a fundamental role in the fermentation of traditional dry sausages. Each processing facility harbors a unique internal flora, including beneficial microorganisms that contribute to fermentation and flavor development, as well as potential spoilage organisms and pathogens. During the production of dry fermented sausages, a variety of chemical and physicochemical changes occur, including dehydration, carbohydrate fermentation, acidification, color development, lipid oxidation, lipolysis and proteolysis, all under the influence of microbial activity. Two main groups of microorganisms are primarily responsible for the key transformations that occur during sausage fermentation and maturation. These include lactic acid bacteria (LAB), particularly *Lactobacillus* spp., and Gram-positive coagulase-negative cocci (CNC), such as *Staphylococcus* and *Kocuria* spp.

Fermentation has a dual function in dry sausage production: it improves the meat's organoleptic properties and ensures its microbiological stability and safety. A major aspect of dry sausage fermentation is the controlled activity of lactic acid bacteria (LAB), which metabolize sugars into lactic acid, leading to a reduction in pH, usually below 5.3. This acidification process creates an unfavourable environment for pathogenic bacteria (Petäjä-Kanninen and Puolanne, 2007; Toldrá and Hui, 2014). In addition to lowering pH, some LAB strains likewise produce bacteriocins, antimicrobial peptides that enhance microbial security. These compounds act specifically against Gram-positive bacteria by disrupting cell membranes, inhibiting cell wall synthesis, interfering with nucleic acid and protein synthesis, and altering enzyme functions. Alongside acidification, lowering water activity (aw) through salting and drying is crucial to guarantee safety and prolong shelf life, as it inhibits spoilage organisms and food-borne pathogens, particularly when aw approaches 0.85 (Barcenilla et al., 2022). Despite the safety features of fermentation and drying, the occurrence of food-borne pathogens in artisanal fermented sausages is still a cause for concern. The detection rates of food-borne pathogens in fermented meat sausages, including *L. monocytogenes, E. coli* O157:H7, *Salmonella* spp., *C. botulinum, S. aureus*, *B. cereus* and *Y. enterocolitica*, have been widely reported in the literature (Ferreira et al., 2007, 2006; Gonzalez-Fandos et al., 2021; Moore, 2004; Santos et al., 2005; Sırıken et al., 2006).

3.2.1 Pathogenic bacteria

Portuguese fermented sausages such as *chouriça de carne* and *alheira* are generally considered by consumers as safe food products. Traditionally produced by small-scale producers or artisans, these sausages are prepared using inherited recipes and techniques that remain largely untouched by modern technological innovation. A defining feature of their production is the absence of starter cultures or additives; instead, fermentation occurs spontaneously at moderate rates and cold temperatures, driven by the naturally occurring LAB present in the raw materials. This spontaneous fermentation process confers distinctive sensory characteristics that enhance the authenticity of those sausages. The combined decrease in water activity (aw) and pH during production plays an important role in inhibiting the growth of pathogenic micro-organisms throughout the manufacturing process.–However, these artisanal methods which are characterized by variability in raw material quality, the absence of standardized procedures and limited technological control, the absence of heat treatment; can also increase the risk of microbiological contamination by food-borne pathogens, this, in turn, may compromise both the safety and overall quality of the final product (Carvalho et al., 2010; Marques et al., 2006; Pavelquesi et al., 2021).

Such uncontrolled processes not only pose health problems but also undermine consumer confidence and may lead to economic losses for the fragile local communities that depend on these traditional foods. According to Omer et al. (2018) and Lücke and Zangerl (2014), pork meat and dry-cured meat products can be contaminated by *Listeria monocytogenes* and *Salmonella*. Other harmful bacteria, such as *C. botulinum*, *C. perfringens*, *S. aureus*, and *E. coli*, have also been detected in dry-cured sausages (Gonzalez-Fandos et al., 2021; Lücke and Zangerl, 2014; Omer et al., 2018).

3.2.2 *Staphylococcus aureus* as a source of infection

S. aureus is recognized as one of the most important foodborne pathogens globally (Wu et al., 2018). Staphylococcal food poisoning (SFP) ranks among the most common food-borne illnesses in the world. The Centre for Disease Control and Prevention (CDC) estimate that in the US, *S. aureus* causes approximately 241,188 illnesses, 1,064 hospitalizations, and 6 deaths each year (Scallan et al., 2011). In 2023, the European Food Safety Authority (EFSA) reported 207 total outbreaks of SFP, with 32 strong evidence, resulting in 113 hospitalisations and one case of deaths (EFSA, 2024).

S. aureus, a prominent member of the genus *Staphylococcus* within the phylum Firmicutes, non-motile, non-capsulated, non-spore-forming bacterium, oxidase-negative, catalase-positive, and coagulase-positive bacterium, (Pal et al., 2020a), is generally part of the normal flora of human and animal skin and mucous membranes (Addis, 2015; Algammal et al., 2020). *S. aureus* is a Gram-positive bacterium recognised in both the clinical and food safety sectors. Microscopically, it usually appears as distinctive grape-like clusters, with cells around 0.8 µm in diameter and has large, round, golden-yellow colonies, typically with haemolysis, when cultured on the blood agar plates (Addis, 2015; Asmare et al., 2019).

This facultative organism is highly resistant, tolerating both drying and high osmotic environments, which enables it to persist on human skin, the nasal passages and various surfaces (Mahendra et al., 2022). It adapts to a wide range of temperatures (7-48.5 °C, optimal at 30-37 °C) and pH levels (4.2-9.3, ideal at 7-7.5) (Chaibenjawong and Foster, 2011; Gardete and Tomasz, 2014). This adaptive capacity enables it to expand in foods with a neutral pH, such as meat, poultry, fish, eggs and milk, which are common sources of bacterial growth (Rho and Schaffner, 2007).

Besides, *S. aureus* produces a broad array of toxins, including staphylococcal enterotoxins, leukocidin, exfoliatin, and toxic shock syndrome toxin 1 (TSST-1). Many of these toxins, particularly the enterotoxins, are heat-resistant and can persist in food even after cooking, leading to food poisoning when contaminated products are consumed (Sugrue et al., 2019). Therefore, it is practically important to prevent *S. aureus* contamination in food because of the high levels of enterotoxins (Liang et al., 2023).

S. aureus can be transmitted between animals and humans in both directions. The direct contact is considered as one of the main modes of transmission, generally via the hands, with colonised or infected individuals, animals and through contaminated objects and surfaces (Pal et al., 2020). *S. aureus* can be transmitted via different pathways (Fig. 2), the most common one includes the transfer from an infected mammary gland to an uninfected gland via fomites, like contaminated milking equipment or the milker's hands, uncontrolled transport of animals between different farms and the handling or ingestion of contaminated food with *S. aureus*. In addition to the direct contact, airborne transmission can represent another possible route of infection since *S. aureus* is present in the nose and on the skin of contaminated people and animals. Furthermore, houseflies (*Musca domestica*) have also been identified as transmission vectors for *S. aureus* (Catry et al., 2010; Cuny et al., 2010).

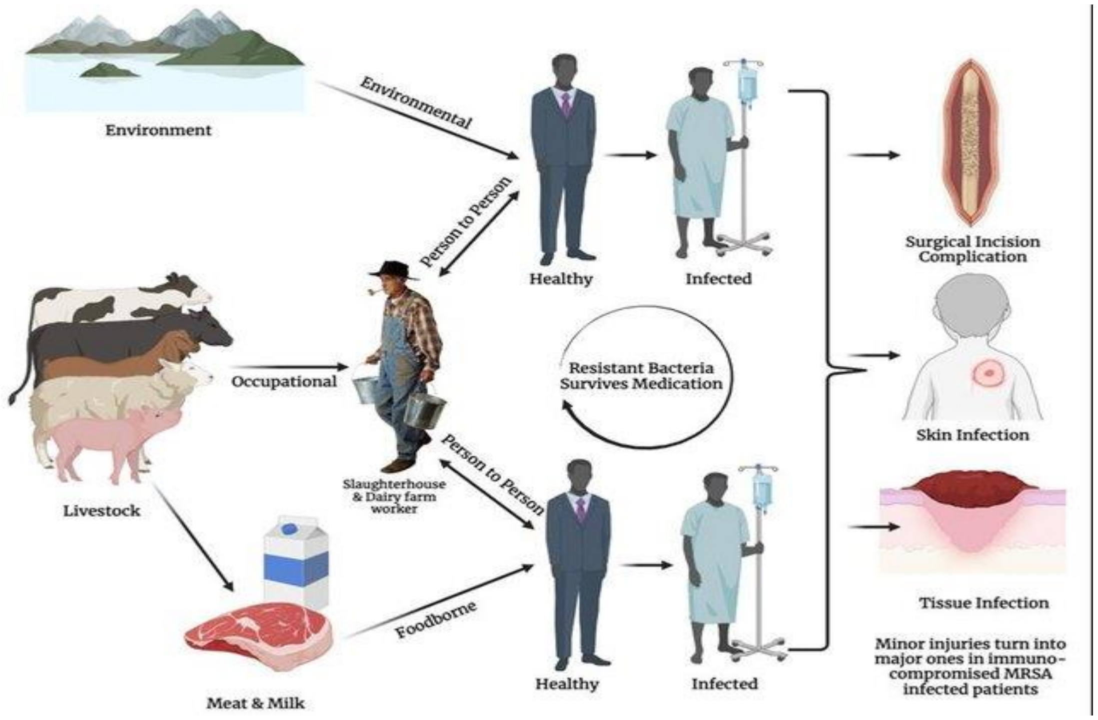


Figure 2: Zoonotic transmission of *Staphylococcus aureus* (Source: Akhtar et al., 2024)

3.3. Public health implications and pathogenic mechanisms of *Staphylococcus aureus*

Although *S. aureus* is a widespread opportunistic pathogen that is part of the natural microbiota of humans and animals (Dini et al., 2019; Pal et al., 2020a; Pollitt et al., 2018; Ramana et al., 2009, 2008), it is also recognised as an invasive human pathogen, responsible for significant morbidity and mortality. Moreover, it is frequently isolated from in animals and animal-derived food products, highlighting its broad presence and impact (Wang et al., 2018).

The spectrum of *S. aureus* infections can vary considerably in terms of severity, from minor skin irritations to life-threatening diseases such as bacteraemia, endocarditis, necrotizing pneumonia, toxic shock syndrome and food poisoning (Lin and Peterson, 2010). Staphylococcal food intoxication occurs primarily when food contaminated with preformed *S. aureus* enterotoxins is ingested, causing acute gastrointestinal symptoms (Argudín et al., 2010). There are five types of *Staphylococcus* enterotoxin (A, B, C, D and E), with enterotoxin A being the main cause of food-borne illness. Between 30% and 80% of people carry *S. aureus*, and half of these strains are linked to food poisoning. Poor hygiene during food handling is a major risk of contamination (Atanassova et al., 2001).

Staphylococcal food poisoning is a worldwide public health problem (Hennekinne et al., 2012), that cause significant morbidity and mortality both in the developing and developed nations (Jackson et al., 2020). Symptoms usually appear early, within 30 minutes to 8 hours (on average 3h) of ingestion of contaminated food (Hennekinne et al., 2010).

Previous studies indicate that the onset of symptoms of staphylococcal food poisoning may depend on the age of the patient. More specifically, children and adolescents appear to have a shorter incubation period than adults (Johler et al., 2015). Key symptoms are nausea, severe vomiting, abdominal cramps and sometimes diarrhoea, moderate fever, and shivering (Fetsch and Johler, 2018). In the case of significant fluid loss, clinical signs of dehydration and hypotension may be manifested (Argudín et al., 2010). Symptoms usually disappear within 24 to 48 hours, but staphylococcal food poisoning can be life-threatening in high-risk populations such as young children, the aged and immunosuppressed individuals (Addis, 2015; Kérouanton et al., 2007).

3.3.1 Virulence factors of *Staphylococcus aureus*

Pathogenic bacteria can detect and adapt to the harsh conditions of the mammalian host during infection, enabling them to infest, colonise and survive regardless of immune defences and antimicrobial treatments (Chakravarty and Massé, 2019). *S. aureus* produces a wide variety of enzymes that enhance its pathogenicity and survival. Coagulase converts plasma fibrinogen into fibrin, which can prevent phagocytosis, while hyaluronidase (spreading factor) breaks down hyaluronic acid in tissues to facilitate spreading (Fig. 3).

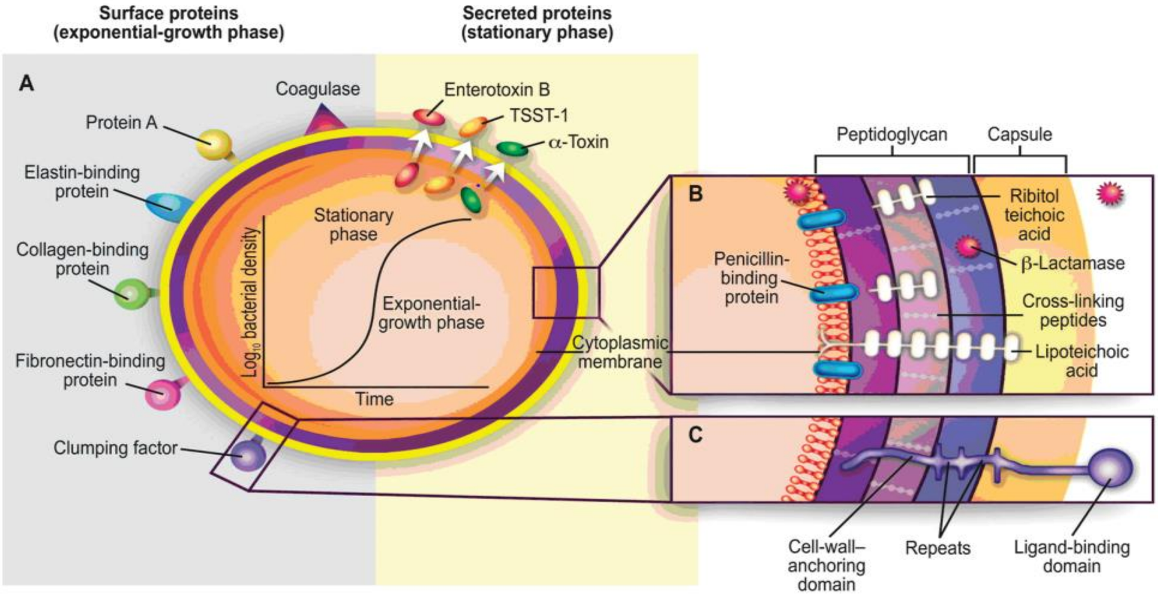


Figure 3: Pathogenic factors of *Staphylococcus aureus*. With structural and secreted products both playing roles as virulence factors. A, Surface and secreted proteins. B and C, Cross-sections of the cell envelope. TSST-1, toxic shock syndrome toxin 1 (Lowy, 1998)

It also produces DNase to degrade DNA, lipase to digest lipids and staphylokinase to dissolve fibrin. In addition, *S. aureus* secretes β-lactamases for antibiotic resistance, as well as esterase, elastase and phospholipase enzymes that facilitate colonisation and infection. Further virulence factors of *S. aureus* include the toxic shock syndrome toxins

(TSST), which provoke excessive lymphokin secretion causing tissue damage, and destruction of immune cells (phagocytes) of some animal species by the production of leucocidin (Quinn et al., 2011).

Depending on the strain, *S. aureus* may secrete a range of toxins, which are key virulence agents. These toxins are grouped into three classes: superantigens, exfoliating toxins and membrane-damaging toxins, including alpha, beta, gamma and delta toxins, as well as bicomponent toxins such as Panton-Valentine leucocidin (PVL) (Foster, 2005; Kashif et al., 2019; Kong et al., 2016).

3.3.2 Antimicrobial resistance of *Staphylococcus aureus*

Antibiotic resistance is becoming a major concern for public health worldwide (WHO, 2014); the bacterial resistance to antibiotics can be either intrinsic or acquired. Intrinsic resistance occurs naturally in resistant bacteria, such as in Gram-negative bacteria, where the lipopolysaccharide (LPS) found in their cell walls provides an innate barrier against the penetration of antimicrobials (Zhang et al., 2013). This type of resistance consists of limiting the absorption of the drug and enhancing its efflux or inactivation (Reygaert, 2018). On the other hand, the acquired resistance occurs via mutations in bacterial genes or the uptake of foreign DNA fragments carrying resistance genes through horizontal gene transfer (HGT) of antibiotic resistance genes (ARGs) (Miller et al., 2014), which allows bacteria to share their genetic material using one of three techniques, including transformation (DNA exchange), transduction (gene transfer by bacteriophage), conjugation with plasmids or transposons (Christaki et al., 2020).

This acquired resistance to antibiotics involve modification of drug targets, activation of drug efflux systems and enzymatic inactivation of antibiotics (Reygaert, 2018). Nevertheless, bacteria have the potential to develop an adaptive mutation in response to the use of antibiotics as a means of resistance (Motta et al., 2015; Munita and Arias, 2016). Previous research has shown that adaptive resistance leads to variations in antibiotic effectiveness, in both laboratory and clinical conditions, leading to antibiotic therapies failure (Christaki et al., 2020). Once bacteria acquire resistance genes through one of the above mechanisms, the genes are expressed different ways, as illustrated in (Fig. 4).

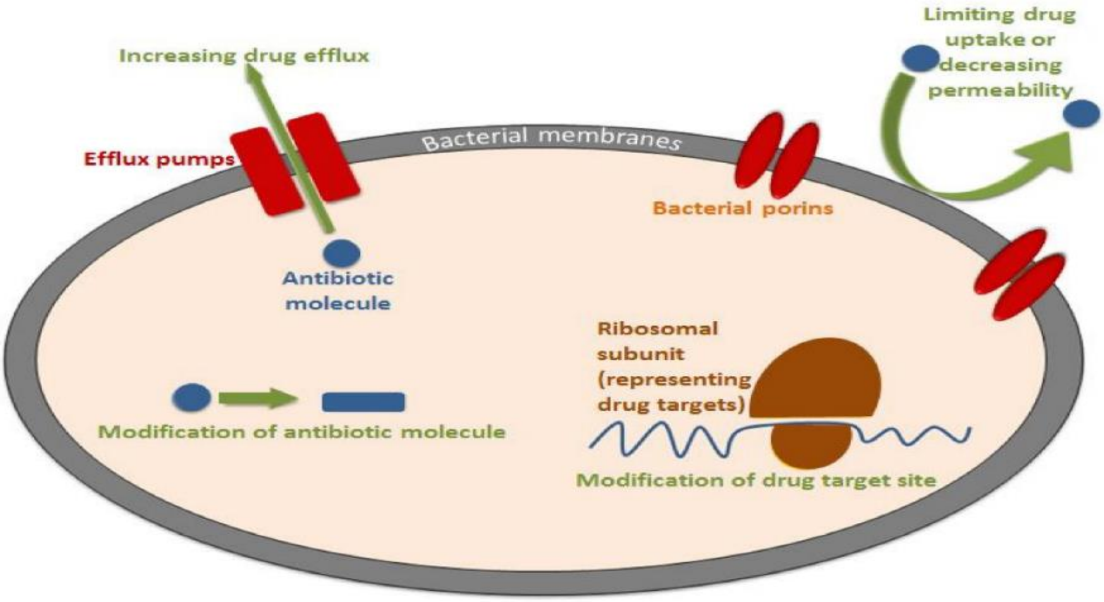


Figure 4: Mechanisms of antimicrobial resistance in bacterial cells (Helmy et al., 2023).

The extensive use of antibiotics has significantly accelerated the evolution of *S. aureus*, enabling it to gain multiple resistance genes (Klare et al., 2003). Consequently, this bacterium has become resistant to nearly all classes of antibiotics, including beta-lactams, macrolides, tetracyclines, fluoroquinolones and aminoglycosides. Notably, *S. aureus* has continued to develop resistance even against more recent antibiotics such as linezolid and daptomycin (Foster, 2017; Fuda et al., 2005). Recent studies demonstrated that in order to minimise the antibiotic resistance issues, an auxiliary compound called thioridazine, which potentiates the effect of dicloxacillin, a β-lactam antibiotic, is used against MRSA (Wassmann et al., 2018).

Staphylococci have developed two major strategies for resisting beta-lactam antibiotics. The first involves producing an enzyme called β-lactamase, which can hydrolyse the antibiotic's structure and render it ineffective. Based on the variations in the hydrolysis of the β-lactam ring, penicillinase can be classified into four different classes: A, B, C and D. The blaZ genes that are responsible for the coding of penicillinase A, C, D, are generally located on the plasmids, while the blaZ that encode for penicillinase B is located on the chromosome (McCallum et al., 2010).

The second approach is to acquire a gene that produces a modified version of a protein called penicillin-binding protein (PBP). This altered PBP is naturally resistant to beta-lactams, enabling the bacteria to persist even in the presence of these drugs (Foster, 2017; Fuda et al., 2005). The emergence of methicillin-resistant *S. aureus* (MRSA) is the outcome of a specific genetic adaptation.

This adaptation includes the acquisition of the mecA gene, which encodes a modified penicillin-binding protein known as PBP2a or PBP2′ (Catry et al., 2010), which are considered as the molecular foundation of resistance to methicillin and oxacillin (Peacock and Paterson, 2015). The *mecA* gene is located within a distinct mobile genetic element known as the staphylococcal cassette chromosome mec (SCCmec) (Liu et al., 2016). The penicillin-binding protein 2a (PBP2a) remains functional despite the presence of β-lactam antibiotics, which inhibit native PBP. This helps the bacteria to continue synthesising its cell wall, ensuring its growth despite the presence of these antibiotics (Llarrull et al., 2009). The expression of the mecA gene is regulated by a two-component system, the mecA repressor gene and mecR1, a transducer-sensory signal protein (McCallum et al., 2010).

Aminoglycosides are efficient antibiotics for treating *S. aureus* infections. Epidemiological studies have shown a high correlation between methicillin resistance and aminoglycoside resistance in *S. aureus* isolates (Thabet et al., 2020). This combination emphasises the clinical challenges posed by MRSA infections, as it still limits the therapeutic options for these MDR pathogens (Tan et al., 2020). The resistance of *S. aureus* to aminoglycosides such as gentamicin, tobramycin and kanamycin is usually associated with the presence of aacA or *aphD* genes. This gene encodes a bifunctional enzyme (AAC6 or APH2), that inactivates these antibiotics, rendering them ineffective. The aacA or aphD gene is generally located on plasmids in MRSA strains (Udou, 2004).

Macrolides (such as erythromycin, azithromycin, and spiramycin) and lincosamides (including clindamycin and lincomycin) are two classes of antibiotics, sharing the same inhibitory effects on bacterial protein synthesis despite their chemical differences. The major mechanism of resistance to macrolides and lincosamides is the inhibition of protein synthesis. There are several possible mechanisms behind this phenomenon, either by the modification of the ribosomal binding site (by methylation or mutation in the 23S rRNA gene) encoded by the erm genes (ermA, ermB, ermC, ermY, and ermF), or through an active efflux mediated by msrA/B genes, although by the inactivation of antibiotics (Mišić et al., 2017). In addition, The inactivation of lincosamide antibiotics is promoted by the activation of lincosamide nucleotidyl transferase enzyme, encoded by the lnu(A) gene (Lina et al., 1999; Lüthje et al., 2007).

Tetracyclines are wide-spectrum antibiotics which are used to treat and prevent bacterial infections in humans and animals and used as growth promotor in livestock (Ardic et al., 2005). There are two main mechanisms that are involved in *S. aureus* resistance to tetracycline, which are the ribosomal protection by elongation of proteins, and activation of the efflux pump.

Ribosomal protection is coded by tetM and tetO genes carried on the chromosome, while the efflux pump is coded by tetK and tetL genes located on plasmids. The resistance mechanism to tetracycline in *S. aureus* has recently been linked to the tet38 gene, which codes for an efflux pump. This pump is responsible for actively expelling tetracycline antibiotics from bacterial cells, thereby reducing their efficacy. The mgrA gene, which produces the MgrA protein, regulates this resistance mechanism. the MgrA protein acts as an indirect inhibitor of the tet38 efflux pump. This means that while the MgrA gene is active, it represses the expression of the tet38 gene, thereby keeping tetracycline resistance under control. However, if the MgrA gene is inactivated or its function is disrupted, this leads to increased expression and activation of the tet38 efflux pump (McCallum et al., 2010).

3.4. The One Health perspective

Antimicrobials have greatly enhanced human and animal health; however, their effectiveness has declined due to overuse in healthcare, communities and livestock. This misuse has led to the emergence of MDR micro-organisms, leading to a global rise in AMR and posing a serious risk to public health by complicating the treatment of disease. In addition, resistant bacteria can enter the food chain, increasing the potential for antimicrobial resistance in foodborne pathogens (Cooper et al., 2020; Thapa et al., 2020).

The lack of effective antimicrobials is leading to a dramatic rise in healthcare costs, as well as an increase in the incidence of disease and mortality rates (OHHLEP et al., 2022; Schneider et al., 2019). The ‘One Health’ approach is a global strategy to promote sustainable health for ecosystems, humans and animals. To control the increase in AMR, the One Health facilitates the intersectoral communication and collaboration networks, enabling the implementation of effective AMR surveillance programmes (Bordier et al., 2018; Mitchell et al., 2020).

In order to reach the objectives of the ‘One Health’ initiative, it is fundamentally important to survey the key elements of AMR transmission that define the pathways by which AMR is spread between humans, animals, plants and the environment (Baquero et al., 2019).

In the pig industry, AMR surveillance must cover the entire value chain, including production, slaughter and processing. Successful mitigation strategies can involve the application of strict hygiene practices throughout the chain, alongside surveillance systems. In addition, promoting biosecurity measures and advancing vaccine research and development are essential elements in reducing the risk of AMR (Qian et al., 2022).

The ‘One Health’ approach, a multi-level system, is challenged by the difficulty of predicting multi-level effects. Informatics has tackled this problem using computational membrane modelling, recently applied to the prediction of antimicrobial resistance. This approach makes it possible to develop advanced biochemical, microbiological, ecological, computational and bioinformatics techniques, which are essential for understanding and controlling antimicrobial resistance on a global scale (Bizzaro et al., 2022; Jeleff et al., 2022; Qian et al., 2022)

IV. MATERIALS AND METHODS

A comprehensive methodology will be carried out based on phenotypic analyses to allow in-depth evaluation of antibiotic resistance in *S. aureus* isolates from traditional dry-fermented sausages that were collected from commercial establishments in Northern Portugal. By focusing on this approach, we anticipate gaining a more complete understanding of the mechanisms and patterns of resistance in *S. aureus* strains.

As illustrated in the diagram below, the methodological approach to exploring the AMR profile of *S. aureus* isolates followed a structured workflow. First, isolates were reactivated from cryopreserved stocks and their purity confirmed. Next, all isolates were phenotypically characterized for antimicrobial resistance, using the Kirby-Bauer disk diffusion method according to internationally recognized guidelines (EUCAST). The dataset was subsequently subjected to both univariate and multivariate statistical analyses to identify patterns of resistance and possible associations between antibiotic resistances.

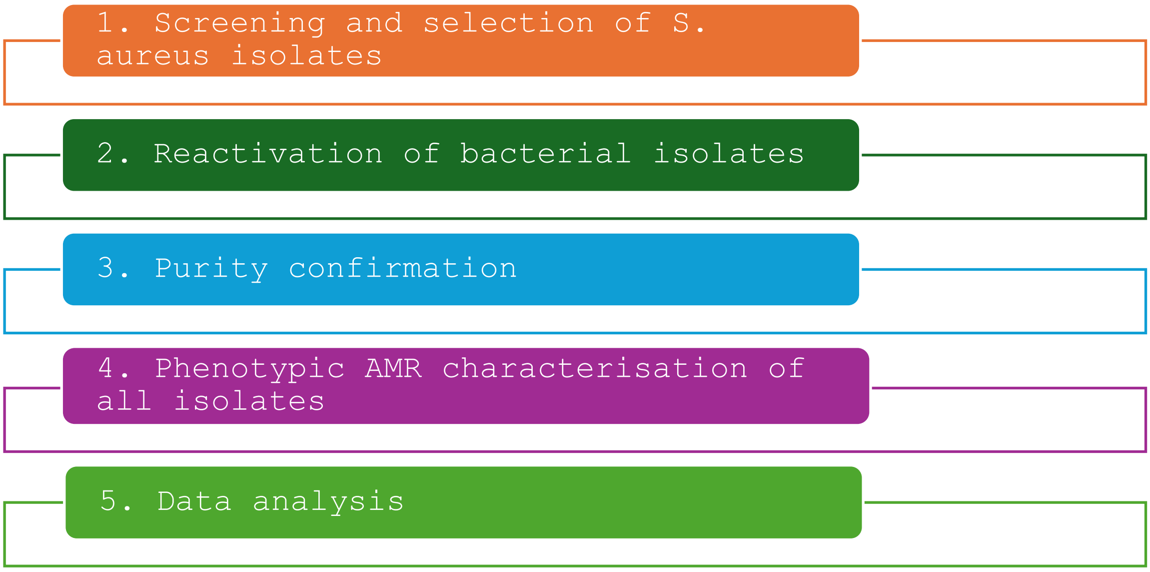


Figure 5: Methodological design established for the development of the current research work

4.1 Test strain selection

A group of 47 isolates of *S. aureus* used in this study were previously recovered from RTE and non-RTE Portuguese fermented sausages and preserved in 30% glycerol cryotubes at −80 °C (Faria et al., 2024).

4.1.1 Preparing Materials for Sterilization

To guarantee aseptic conditions, all necessary laboratory supplies were gathered and sterilized before purifying *S. aureus* isolates. This comprised Petri plates, boxes of micropipette tips, microcentrifuge tubes, and glass bottles used to prepare culture media. An autoclave was used to sterilize for 15 minutes at 121 °C. This process guaranteed that all supplies were free of contaminants, preventing cross-contamination during culture handling.

- **Culture medium preparation**

- Brain Heart Infusion (BHI) Broth

For the preparation of BHI, 37 g of powder was dissolved in 1 L of distilled water. The mixture was stirred thoroughly until the powder was completely dissolved, then 10ml of the medium was distributed in sterilized glass tubes and autoclaved. Once cooled to room temperature, the sterile BHI broth was ready to be used for the activation and cultivation of *S. aureus* isolates or stored at 4°C until use.

- Baird Parker Agar

For Baird-Parker agar, 63 g of dehydrated medium were suspended in 1 L of distilled water and heated with frequent agitation until completely dissolved. After autoclaving, the agar was cooled to around 45-50°C before the addition of 50 ml of egg yolk tellurite emulsion supplement. The medium is then poured into sterile Petri dishes under aseptic conditions, left to solidify, and stored at 4°C until use. Baird-Parker agar is a differential; selective culture medium designed specifically for the isolation and identification of *S. aureus*. This medium inhibits the growth of most competing species, while allowing *S. aureus* to develop as typical black, shiny, convex colonies, often surrounded by bright halos due to lecithinase activity. These visual characteristics help distinguish *S. aureus* from other staphylococci and contaminants during colony selection and purification steps.

- Nutrient Agar medium

Nutrient Agar (NA) is a non-selective medium that allows the growth of a wide range of non-fastidious bacteria. For the preparation of NA, 28 g of powder was dissolved in 1 L of distilled water. The mixture was stirred and gently heated until the powder was completely dissolved. The medium was then dispensed into glass bottles or flasks, sealed, and autoclaved. Afterwards, the medium was allowed to cool to approximately 45–50°C before being poured into sterile Petri dishes under aseptic conditions. Once solidified, the plates were stored at 4°C until use.

4.1.2 Reactivation of bacterial isolates

For bacterial cell reactivation, using a sterile loop, 100 µl of defrosted cryopreserved culture was inoculated into 10 ml of sterile BHI broth and incubated at 37°C for 16-18 h under aerobic conditions.

4.1.3 Purity confirmation

After overnight incubation, the bacterial suspension was inoculated onto Baird-Parker agar plates to recognize the specific morphology of *S. aureus* colonies The plates were incubated at 37 °C for 24–48 hours. Presumptive *S. aureus* colonies were typically black, shiny, and convex with clear zones (indicative of lecithinase activity on egg yolk). In cases where mixed bacterial growth was observed, a single, well-isolated colony with typical *S. aureus* characteristics was subcultured onto a fresh Baird-Parker agar plate to obtain a pure culture (Fig. 6). This purification step was repeated as necessary to ensure clonal purity.

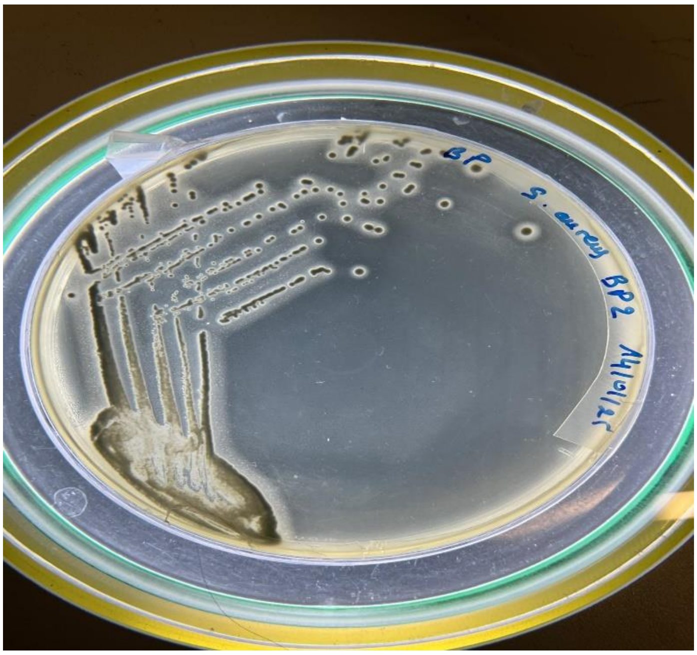


Figure 6: Typical colonies *Staphylococcus aureus* on Baird-Parker agar

A well-isolated and morphologically typical colony of *Staphylococcus aureus* was selected from a Baird-Parker agar plate. Using a sterile inoculating loop, the colony was carefully transferred and streaked onto a freshly prepared NA plate under aseptic conditions. The NA plate was then incubated at 37°C for 24 hours to allow for bacterial growth. This step was essential to obtain a clean and abundant culture for further phenotypic analyses (Fig. 7).

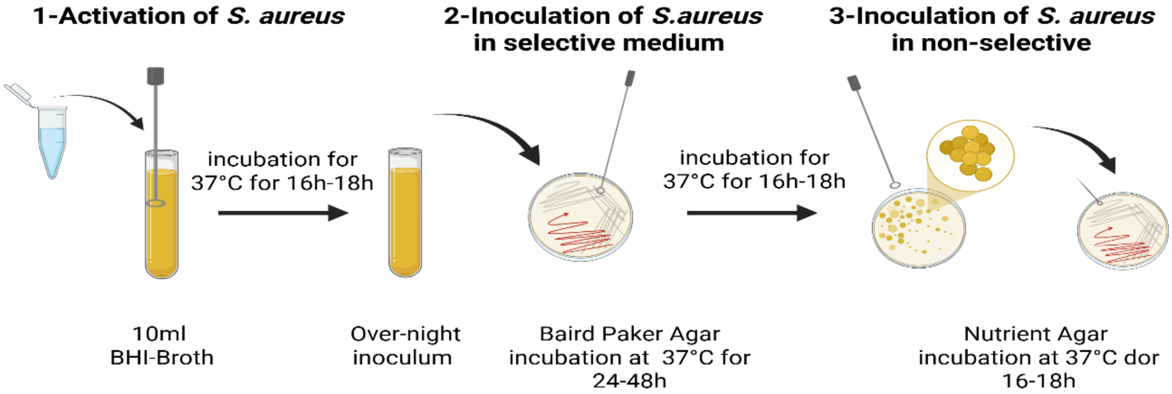


Figure 7: Experimental procedure for *Staphylococcus aureus* activation and purification

4.2 Phenotypic Characterization of *Staphylococcus aureus* Isolates

After obtaining a pure culture, the isolates of *S. aureus* were subjected to antibiotic susceptibility testing using the Kirby-Bauer disk diffusion method as per the recommendation of the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2025). This method was chosen because it is considered the most commonly used approaches for antimicrobial susceptibility testing (AST) in routine clinical microbiology laboratories (Matuschek et al., 2014). When conducted according to standard recommendations, the disk diffusion method is known for its reproducibility and accuracy in assessing antimicrobial resistance (Jones, 1992; Woods, 1995).

4.2.1 Preparation of media

Non-supplemented MH agar is used for non-fastidious organisms; 38 g of the dehydrated medium was suspended in 1 L of distilled water. The mixture was stirred thoroughly until the powder was completely dissolved. The medium was then dispensed into glass bottles or flasks, sealed, and autoclaved. The agar was then dispensed in Petri dishes to achieve an even depth of 4.0 mm with a maximum variation of ±0.5 mm. Once solidified, the plates were stored at 4-8°C until use.

4.2.2 Preparation of inoculum suspension

Multiple colonies of *S. aureus* were first selected from an overnight culture (16–24 hours of growth) on a non-selective agar medium (Nutrient Agar) for the preparation of the inoculum suspension.

Using a sterile loop, multiple colonies with identical morphological appearance were gently collected to avoid selecting atypical variants and then were suspended in sterile saline water (0.9% NaCl) and mixed thoroughly to createa uniform bacterial suspension. The turbidity of the suspension was adjusted to match the McFarland 0.5 standard, equivalent to approximately 1–2 × 10^8 colony-forming units per millilitre (CFU/mL) for *Escherichia coli*. This standardization was performed using a calibrated photometric device in a range of absorbance at 625 nm = [0.08 - 0.13] to ensure accuracy, following the manufacturer's guidelines. If needed, additional saline or bacterial cells were added to achieve the target turbidity. This step ensured consistent bacterial density across tests, a critical requirement for reliable disk diffusion antibiogram results. All inoculum suspensions should optimally be used within 15 min and always within 60 min of preparation.

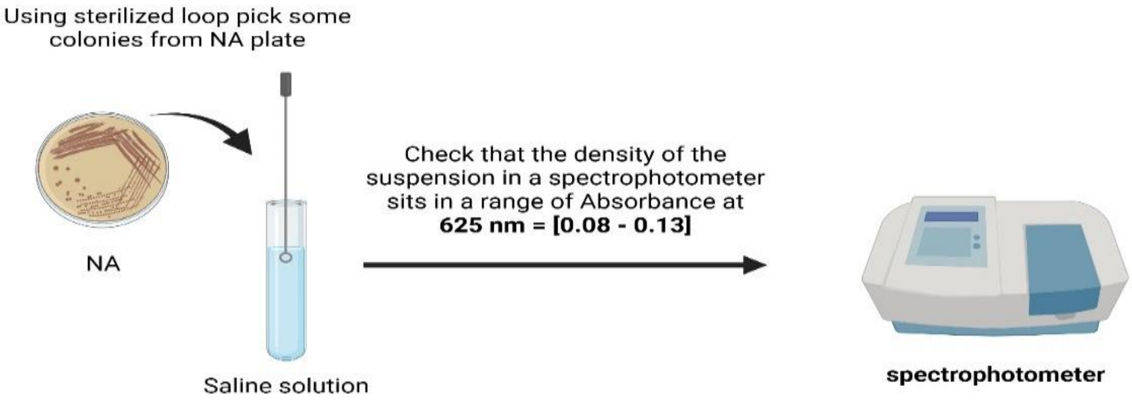


Figure 8: Preparation of standardised bacterial inoculum for antimicrobial testing

4.2.3 Inoculation of agar plates

A prior MH plate drying is required to avoid excess of moisture that may cause fuzzy zone edges and haze within zones. Thus, plates were dried either at 20-25°C overnight, or at 35°C, with the lid removed, for 15 min, without over-drying. A sterile cotton swab is dipped into the inoculum suspension, then the inoculum is spread evenly over the entire surface of the agar plate by swabbing in three directions or by using an automatic plate rotator.

4.2.4 Application of antimicrobial disks

Antibiotic disks were handled in strict accordance with the manufacturer's specifications to preserve stability and antimicrobial efficacy.

After inoculation of the MH agar, fourteen antibiotic disks of known concentrations (benzylpenicillin (PEN) (1U), cefoxitin (FOX) (30 µg), norfloxacin (NOR) (10 µg), amikacin (AMK) (30 µg), gentamicin (GEN) (10 µg), tobramycin (TOB) (10 µg), erythromycin (ERY) (15 µg), clindamycin (CLI) (2 ug), tetracycline (TCY) (30 µg), linezolid (LZN) (10 µg), rifampicin (RIF) (5 µg), trimethoprim (TMP) (5 µg), trimethoprim-sulfamethoxazole (SXT) (1.25-23.75 µg) and chloramphenicol (CHL) (30 µg) were aseptically applied on the agar within 15 minutes of inoculation to prevent premature absorption of moisture and ensure consistent diffusion kinetics.

For a given strain, each antibiotic was tested in triplicate in order to ensure the reliability of the experiment and the reproducibility of the results, with disks positioned using sterile forceps under laminar airflow conditions. To avoid overlapping inhibition zones and ensure accurate measurement, the disks were placed at a minimum distance of 24 mm from centre to centre, as stipulated by the standard antibiogram protocol (EUCAST, 2025). The number of disks per plate was optimised according to plate dimensions (e.g., ≤6 disks per 90 mm plate) and strain-specific growth characteristics, maintaining methodological consistency between replicates. For the detection of inducible clindamycin resistance, the erythromycin and clindamycin disks were placed 12–20 mm apart (edge to edge) on the agar surface. This arrangement allows for observation of the D-shaped zone of inhibition (D-test), which indicates antagonism and the presence of inducible resistance to clindamycin (Fig. 9)

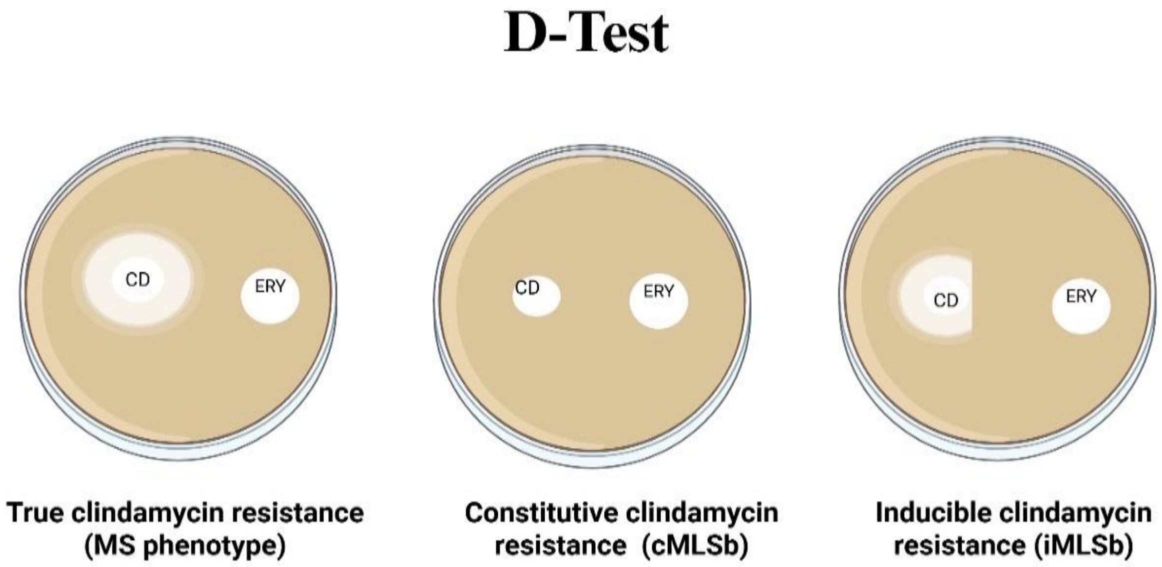


Figure 9: Representative D-Test patterns for detecting clindamycin resistance phenotypes in *Staphylococcus aureus*

Table 1: Antimicrobial susceptibility testing breakpoints and quality control ranges for *Staphylococcus aureus* (Disk Diffusion Method) (EUCAST, 2025)

							QC strain	
				Zone diameter breakpoints (mm)		**ATU**	**Inhibition zone diameter (mm)**	
Antibiotic Classes		**Antibiotic**	**Disk content (µg)**	**S ≥**	**R <**		**Target**	**Range**
Penicillins	**1**	**Benzilpenicillin/Penicillin G** (*S. aureus*)	1 unit	**26**[A, B]	26		15	12-18
Cephalosporins	**1**	**Cefoxitin (screen only)**	30	**22**[B]	22		27	24-30
	2	Ceftaroline	5	20	17	19-20	27	24-30
	2	Ceftaroline	5	20	20	19-20	27	24-30
	2	Ceftobiprole	5	17	17	16-17	25	22-28
Carbapenems		**Infer susceptibility to Carbapenems from Cefoxitin susceptibility.**					–	–
Monobactams		–	–	–	–		–	–
Fluoroquinolones	**1**	**Norfloxacin**	10	17	17		21	18-24
	2	Ciprofloxacin (*S. aureus*)	5	(50)	(17)		24	21-27
	2	Moxifloxacin (*S. aureus*)	5	25	25		28	25-31
Aminoglycosides	**1**	**Amikacin**	30	(15)	(15)		21	18-24
	1	**Gentamicin** (*S. aureus*)	10	(18)	(18)		22	19-25
	1	**Tobramycin** (*S. aureus*)	10	(18)	(18)		23	20-26
Macrolides, lincosamides, and streptogramins	**1**	**Erythromycin**	15	**21**[C]	**21**[C]		26	23-29
	1	**Clindamycin**	2	**22**[C]	**22**[C]		26	23-29
Tetracyclines	**1**	**Tetracycline**	30	22	22		27	23-31
	2	Tigecycline	15	19	19		22	19-25
Oxazolidinones	**1**	**Linezolid**	10	21	21		24	21-27
Miscellaneous agents	**1**	**Rifampicin** (*S. aureus*)	5	26	26		33	30-36
	1	**Trimethoprim**	5	17	14		25	22-28
	1	**Trimethoprim-sulfamethoxazole**	1.25-23.75	17	14		29	26-32
	1	**Chloramphenicol**	30	18	18		24	20-28

QC: Quality control

ATU: Area of Technical Uncertainty

A- Check zone edge: ≥26mm & sharp edge = Resistant; ≥26mm & fuzzy edge = Susceptible.

B- Isolates a) Cefoxitin resistant = resistant to all Penicillins; b) cefoxitin susceptible + Penicillin G resistant = susceptible to isoxazolylpenicillins (oxacillin, cloxacillin, etc); c) cefoxitin susceptible + Penicillin G susceptible = susceptible to all penicillins.

C- Place the erythromycin & clindamycin disks 12-20 mm apart & look for antagonism to detect inducible clindamycin resistance.

S: Susceptibility; R: Resistance.

4.2.5 Incubation of plates

After application of the antibiotic disks, the agar plates were inverted to avoid any condensation and to ensure that the disks would not fall off the agar surface. Plates were incubated within 15 minutes of disk placement to maintain optimal diffusion conditions. To prevent uneven heating, which could affect the accuracy of results, plates were stacked no more than five high in the incubator. Incubation was carried out at 35 ± 1°C in ambient air for 16-20 h, according to the standard guidelines for antimicrobial susceptibility testing.

4.2.6 Examination of plates after incubation

Following incubation, each agar plate was examined to confirm the development of a uniform, confluent bacterial lawn, indicating an appropriate inoculum density and streaking technique. Plates showing isolated colonies, irregular growth, or ragged inhibition zone edges were excluded from analysis, as these features suggest insufficient inoculum or sub-optimal seeding. Only plates with homogeneous growth were considered valid for the measurement of inhibition zone diameters.

4.2.7 Measurement of inhibition zone diameters and interpretation of results

The inhibition zone was evaluated by precisely measuring the diameter of the clear area surrounding each antibiotic disk, where no bacterial growth was observed. Measurements were taken to the nearest millimetre using a calliper. Non-supplemented MH agar plates were read from the back of the plate with reflected light against a dark background. For antibiotics producing double zones of inhibition, the inner zone was considered for the measurement, unless otherwise specified in the interpretation guidelines. Specific reading instructions are given in the EUCAST disk diffusion test manual and the EUCAST reading guide. The results were then analysed and classified as sensitive or resistant according to the breakpoints defined in the EUCAST clinical breakpoint tables (EUCAST, 2025) (Fig. 9).

4.2.8 Quality control

The reference strain *S. aureus* ATCC 29213 was used as a quality control standard to validate the antibiogram of *S. aureus* isolates. To maintain accuracy and reliability, quality control tests for commonly used antimicrobial agents were ideally performed routinely. In addition, each new batch of MH agar (i.e., a different batch (new lot) of agar powder) prepared in-house was tested with the control strain to assure that the inhibition zone diameters obtained were within the acceptable ranges established by EUCAST quality control guidelines.

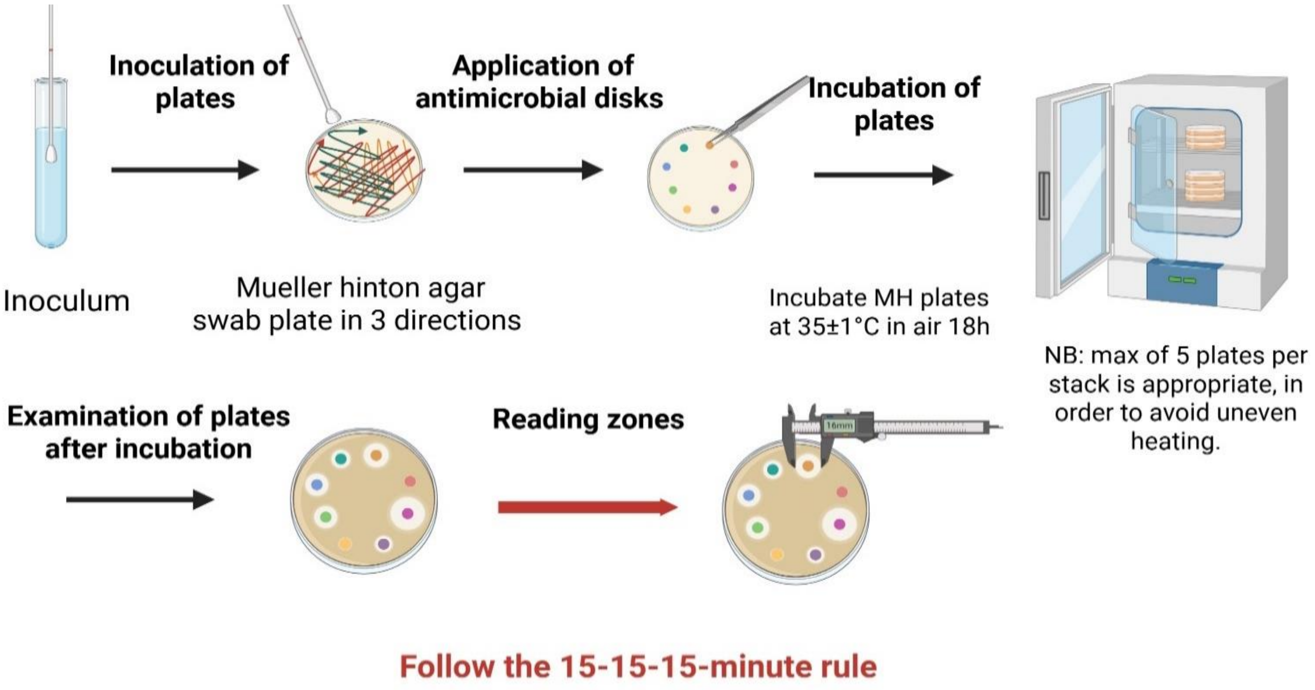


Figure 10: Disk diffusion method protocol (Kirby-Bauer) for antimicrobial susceptibility testing of *Staphylococcus aureus* (EUCAST protocol)

4.3 Statistical analysis

The data on the antibiotic-resistance profile obtained from the subset of 47 isolates recovered from Portuguese RTE and non-RTE sausages were divided into two main parts: **descriptive analysis (**univariate**)** and **Principal Component Analysis (**multivariate**)**. All analyses were performed using R statistical software version 4.4.2.

4.3.1 Descriptive analysis

Descriptive analyses were performed to characterise the antibiotic resistance profiles of the isolates. These analyses involved calculating the frequency of resistance to each 14 antibiotics, creating summary tables and graphs, and constructing correlation matrices to evaluate the potential relationship between the antibiotic classes. The objective of this analysis was to identify the most common resistance traits by production zone and by RTE status; and highlight potential co-resistance relationships between isolates.

4.3.2 Principal component analyses

Principal component analysis (PCA) was performed to reduce the dimensionality of the dataset and to investigate underlying patterns and groupings in antibiotic resistance profiles. The first three principal components were selected for further analysis, as they together accounted for about 60% of the total variance in the antibiotic resistance data. Two-dimensional PCA plots were generated to visualize the distribution of *S. aureus* isolates according to RTE status, and production zone. This multivariate approach provided valuable insights into potential groupings of resistance patterns, which might be associated with specific antibiotic classes or sources of isolates.

V. RESULTS AND DISCUSSION

Antimicrobial resistance (AMR) represents a serious challenge to public health worldwide and in Portugal, limiting the effectiveness of antimicrobial therapies. This report presents the phenotypic resistance profiles of Staphylococcus aureus isolates from traditional Portuguese sausages. For the completeness and transparency of the report, the full datasets, including detailed tables and illustrative figures, is provided in the appendix to support and complement the findings discussed.

5.1 Prevalence of single antibiotic resistance

Table 2 below revealed the phenotypic analysis of antibiotic resistance among forty-seven *S. aureus* isolates, obtained from two types of Portuguese fermented sausages, chouriça (RTE, n=22) and alheira (non-RTE, n=25). They showed significant differences in resistance profiles, while 68.08% (32/47) of strains showed antimicrobial resistance properties to at least one of the antibiotics tested (Fig. 11, Table 2).

S. aureus isolates from both types of sausages harbored the highest prevalence of resistance to the antibiotic of PEN (RTE, 50%; non-RTE, 40%). ERY resistance was also prominent, particularly among RTE isolates (40.9%), compared to a lower prevalence in non-RTE (16%). TCY resistance was the least common across both food types, accounting for 9% of RTE and 4% of non-RTE isolates. NOR resistance was observed exclusively in non-RTE isolates (16%), while modest levels of resistance to CLI, TOB (9%), TMP, and GEN (4.5%) were only detected in RTE isolates.

However, all 47 *S. aureus* strains were susceptible to FOX, AMK, LNZ, RIF, SXT, and CHL. These data show both common and distinct patterns of antibiotic resistance in *S. aureus* associated with different types of fermented sausages.

Research has revealed that *S. aureus* strains isolated from various food products exhibited resistance to a wide spectrum of antibiotics (Kukułowicz et al., 2025; Moges et al., 2024; Szczuka et al., 2022). β-lactam antibiotics, such as PEN and methicillin, inhibit bacterial cell wall synthesis by targeting penicillin-binding proteins (Ali et al., 2017). However, overall resistance to these antibiotics in *S. aureus* has increased substantially in recent years (Arefi et al., 2014).

Table 2: Numbers and percentages of *Staphylococcus aureus* isolates resistant to different antimicrobials production zone and RTE status

RTE status	Production zone	N of. IST	PEN (%)	FOX (%)	NOR (%)	AMK (%)	GEN (%)	TOB (%)	ERY (%)	CLI (%)	TCY (%)	LIZ (%)	RIF (%)	TMP (%)	SXT (%)	CHL (%)
RTE	Vinhais	4	25	0	0	0	0	0	25	25	0	0	0	0	0	0
	Mogadouro	6	66.7	0	0	0	0	0	16.7	16.7	16.7	0	0	0	0	0
	Mirandela	3	100	0	0	0	33.3	33.3	33.3	0	33.3	0	0	33.3	0	0
	Braganza	8	25	0	0	0	0	0	62.5	0	0	0	0	0	0	0
	Miranda do Douro	1	100	0	0	0	0	100	100	0	0	0	0	0	0	0
	Total	22	50	0	0	0	4.55	9.09	40.9	9.09	9.09	0	0	4.55	0	0
Non-RTE	Vinhais	12	83.3	0	8.33	0	0	0	25	0	8.33	0	0	0	0	0
	Braganza	9	0	0	0	0	0	0	11.1	0	0	0	0	0	0	0
	Mirandela	3	0	0	66.7	0	0	0	0	0	0	0	0	0	0	0
	Valpacos	1	0	0	100	0	0	0	0	0	0	0	0	0	0	0
	Total	25	40	0	16	0	0	0	16	0	4	0	0	0	0	0
Total		**47**	**44.7**	**0**	**8.5**	**0**	**2**	**4**	**23**	**2**	**6**	**0**	**0**	**2**	**0**	**0**

Norfloxacin (NOR); Amikacin (AMK); Gentamycin (GEN); Tobramycin (TOB); Erythromycin (ERY); Clindamycin (CLI); Tetracycline (TCY); Linozolid (LIZ); Rifampicin (RIF); Trimethoprim (TMP); sulfamethoxazole-trimethoprim (SXT); chloramphenicol (CHL).

Our study revealed a high prevalence of PEN resistance, aligning with previous research reporting rates of [56.7-73%] in *S. aureus* isolates from various food sources, which is not surprising given the high frequency of β-lactam prescriptions in veterinary (ÇetiNkaya and Mus, 2012; Gonzalez et al., 2017; Pereira et al., 2009; Umoh and Odoba, 1999). More recent research, report even higher resistance rates: 84.71%, 77.9% and 100%, respectively (Kayili and Sanlibaba, 2020; Pereira et al., 2018; Safarpoor Dehkordi et al., 2017). This widespread resistance is primarily associated with the β-lactamase synthesis, which attacks the β-lactam ring.

Related to β-lactam antibiotic class resistance, MRSA is nowadays the most emergent issue. The complete susceptibility of all isolates to cefoxitin indicates the absence of MRSA, as cefoxitin resistance serves as a reliable phenotypic marker for MRSA detection. A review by (Ou et al., 2017) reported that the prevalence of MRSA contamination in meat products was lower in Europe (3.2%) compared to other continents. Likewise, (Islam et al., 2019) detected MRSA in only 5% of RTE food samples contaminated by *S. aureus.* These results suggest relatively low MRSA occurrence in European meat products, though a recent Italian study detected 25% of MRSA strains isolated from meat as raw material (Pinamonti et al., 2025).

ERY resistance was observed in 23.4% of meat product samples screened, comparable to rates reported by Kayili and Sanlibaba (22.35%) but higher than those found in Taiwan (Kayili and Sanlibaba, 2020; Wang et al., 2019). In opposite, (Safarpoor Dehkordi et al., 2017) and(Arefi et al., 2014) reported a much higher erythromycin resistance (100%) and (44%), respectively.

TCY are commonly used in veterinary medicine, particularly in cattle, due to their broad-spectrum activity and low cost, which can explain the prevalence of strains resistant to this antibiotic (Liu et al., 2017; Rola et al., 2016). In our findings, a lower incidence of TCY resistance (6.4%) was observed, consistent with Portuguese research reporting a very low rate (0.7%) across various sources, including raw meat, Portuguese fermented meat products, bovine mastitis cases, and other food products (Pereira et al., 2009). In contrast, higher incidence of TCY resistance 44%, 17% and 100% were reported in various food products, including dairy products and hospital food (Arefi et al., 2014; Pereira et al., 2018; Safarpoor Dehkordi et al., 2017). An Italian study, focused on *S. aureus* isolated from raw milk, beef hamburgers and pork sausages, reported that some of the MRSA strains exhibited a high TCY resistance above (25% in meat and 5.7% in milk) (Pinamonti et al., 2025).

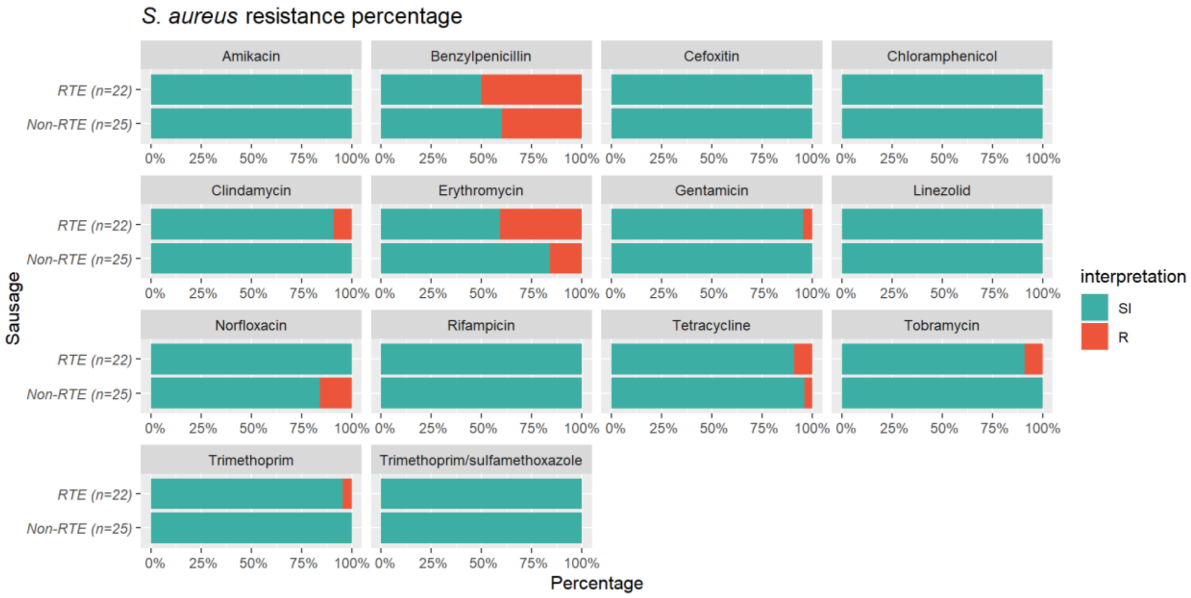
Lincosamide antibiotics, such us CLI and lincomycin are also commonly used as first line for treating animal infections (Jamal et al., 2017). Our finding detects low resistance rate to CLI (2.12%), aligning with studies reporting rates (2-11.1%) (Bulajic et al., 2017; ÇetiNkaya and Mus, 2012; Matallah et al., 2019; Zehra et al., 2019). This contrasts sharply with the higher rates of 40.7% in 54 isolates detected in RTE in bulk (RTEIB food) and 50.59% (43/85 isolates) detected in traditional Turkish cheese, respectively (Kayili and Sanlibaba, 2020; Lin et al., 2019).

Aminoglycoside resistance was also low: GEN (2.12%) and TOB (4.12%), although at slightly lower levels, and no resistance to amikacin was detected. A finding conducted on 160 samples from two meat-processing in Greece reported a resistance rate of 13.6% to TOB and no resistance to GEN and AMK (Komodromos et al., 2022). Likewise, no resistance to GEN was detected in *S. aureus* strains isolated from pork meat in Italy, dairy products in Algeria, cheese products in Brazil, and from food handlers' hands in Malaysia (Gonzalez et al., 2017; Matallah et al., 2019; Pinamonti et al., 2025; Tan et al., 2014, respectively). The injectable formulation of GEN may contribute to its lower resistance prevalence compared to oral antibiotics. In contrast, other studies reported moderately higher resistance rates to GEN (13%-40%) in various food products, including traditional cheeses, raw chicken meat, RTE foods, and bulk tank milk, as well as among livestock and dairy farm personnel (Kayili and Sanlibaba, 2020; Lika et al., 2021; Lin et al., 2019; Papadopoulos et al., 2019).

NOR resistance rate was 8.5% in this study, higher than the lower rates reported from various food sources in Turkey (2.38–3.9%) (Kayili and Sanlibaba, 2020; Yucel et al., 2011). TMP resistance was low (2.12%), in agreement with a study reported resistance rate of 2.35% in traditional cheese products (Kayili and Sanlibaba, 2020), yet lower than rates detected in Portuguese and Greek raw milk products (13%) (Oliveira et al., 2022; Papadopoulos et al., 2019).

It is noteworthy mentioning that all isolates were susceptible to LIZ, RIF, CHL, and SXT. This aligns with studies confirming LIZ and CHL susceptibility from foods of animal origin (Kayili and Sanlibaba, 2020; Oliveira et al., 2022; Yucel et al., 2011). However, other findings reported variable resistance rates for RIF (1.6-21.18%), SXT (3.53-11.1%), and CHL

(1.1-61.77%), across multiple food sources (Kayili and Sanlibaba, 2020; Komodromos et al., 2022; Lika et al., 2021; Lin et al., 2019; Moges et al., 2024; Papadopoulos et al., 2019; Pinamonti et al., 2025). Notably, Pinamonti et al. (2025) reported 8.3% LIZ resistance in *S. aureus* isolated from Italian pork sausages, contrasting with our findings despite linezolid's status as a newer oxazolidinone antibiotic.



a)

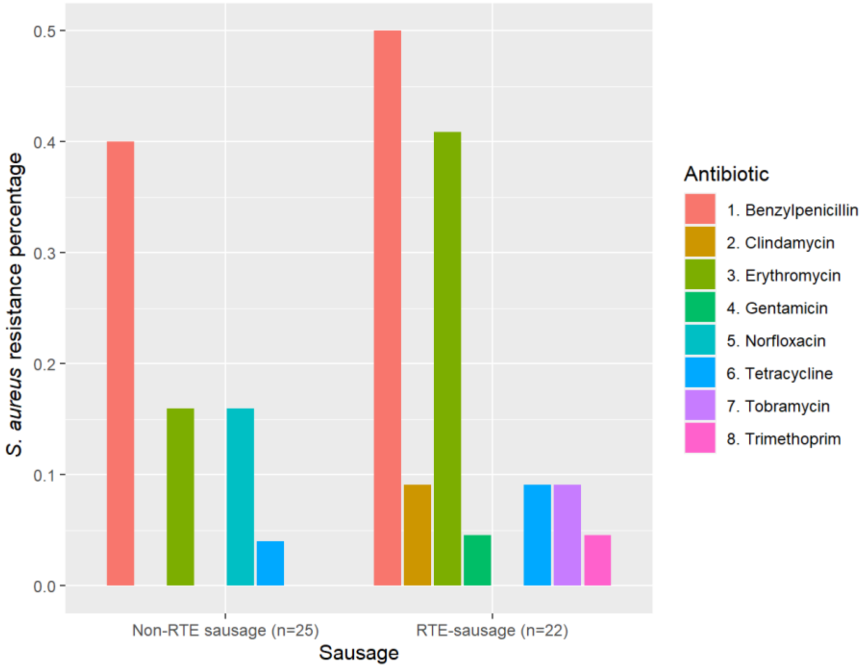


Figure 11 : Percentage of *Staphylococcus aureus* isolates resistant to tested antibiotics

SI: Susceptibility; R: Resistance. Penicillin (PEN); norfloxacin (NOR); amikacin (AMK); gentamycin (GEN); tobramycin (TOB); erythromycin (ERY); clindamycin (CLI); tetracycline (TCY); linozolid (LIZ); rifampicin (RIF); trimethoprim (TMP); sulfamethoxazole-trimethoprim (SXT); chloramphenicol (CHL).

5.2 Isolates with multiple antibiotic resistance

Out of the 47 *S. aureus* isolates recovered from traditional Portuguese sausages, eight isolates (17%) exhibited resistance to at least two antibiotics. Multidrug resistance (MDR), defined as resistance to three or more antimicrobial agents, was found in 3 isolates (6.4%) in this study. Notably, 5 strains sourced from non-RTE sausages (10.6%) showed resistance to exactly two antibiotics, whereas no MRSA strains were detected. This finding underscores the necessity of an accurate and active control against antibiotic resistance in food, particularly on meat, and in community, and nosocomial infections, in order to detect any expansion or increment of new antibiotic resistance that could be transferred to other bacteria. A summary of antimicrobial resistance among *S. aureus* strains is shown in Table 3.

Table 3 : Multiple antibiotic resistance in *Staphylococcus aureus* strains

Food_Source	**Bacterium**	**Isolate code**	**AMR pattern**
Chourica	*Staphylococcus spp.*	**PDO1**	PEN-TOB-ERY
Chourica	*Staphylococcus aureus*	**ML1**	PEN-GEN-TOB- ERY-TCY-TMP
Alheira	*Staphylococcus aureus*	V3	PEN-NOR
Alheira	*Staphylococcus aureus*	FN4(A)	NOR-MFX
Alheira	*Staphylococcus aureus*	FN5(A)	NOR-MFX
Alheira	*Staphylococcus aureus*	**AV2-4D7**	PEN-ERY-TCY-TGC
Alheira	*Staphylococcus aureus*	AV3-2D2	PEN-ERY
Alheira	*Staphylococcus aureus*	AV3-3	PEN-ERY

Penicillin (PEN); norfloxacin (NOR); gentamycin (GEN); tobramycin (TOB); erythromycin (ERY) ; clindamycin (CLI); tetracycline (TCY); trimethoprim (TMP), moxifloxacin (MFX).

Among the RTE sausage-sourced strains, two resistant isolates were identified: **PDO1**, which was resistant to PEN, TOB, ERY; and **ML1**, which showed resistance to six antibiotics: PEN, GEN, TOB, ERY, TCY and TMP. Among the non-RTE sausage-sourced isolates, only one isolate (**AV2-4D7**) met the MDR criteria and was resistant to four classes of antibiotics: PEN, ERY, TCY and TGC.

In agreement with our results, studies from Greece and Italy reported similarly low MDR rates, with only 2 out of 22 and 2 out of 12 *S. aureus* isolates from pork meat products classified as MDR, while a study in the United States found a 2.9% prevalence of MDR MRSA isolates in turkey and pork meat. An Algerian survey performed on RTE food reported a rate of 16.7% of MDR, which still is relatively low compared to other findings (Ge et al., 2017; Komodromos et al., 2022; Pinamonti et al., 2025). In contrast, research conducted in Egypt found that 75% of *S. aureus* isolates obtained from luncheon and minced meat samples were MDR (Helal F et al., 2024), while a Chinese research reported that 28 isolates were resistant to eight or more classes of antimicrobials, among them, with most (92.8%) originating from raw meats and four isolates resistant to ten antibiotic classes (Wang et al., 2017).

An Italian study on poultry, beef and pork meat reported that 13 out of 42 isolates (30.95%) were resistant to all 12 tested antibiotics (Pesavento et al., 2007). Research from South Africa revealed that all tested isolates from sheep and pork were MDR, in particular to PEN, CLI, ERY, and TCY (Pekana and Green, 2018). A recent study on raw chicken meat samples showed that all the positive samples for *S. aureus* isolates (34 isolates) exhibited potential dissemination of MDR to at least 3 antibiotics (Lika et al., 2021).

In comparison to studies conducted on dairy products (milk, cheese) from various countries, the MDR patterns reported in other countries were as follows: 72.94% in Turkey, 50% in Serbia, 29% in Brazil, 57.1% in Tanzania, 61.1% in China, 21.74% in Iran and 66.67% in USA. These differences in MDR prevalence among *S. aureus* strains isolated from traditional cheese and milk products may be attributed to regional variations in antibiotic usage practices (Arefi et al., 2014; D'amico and Donnelly, 2011; Kayili and Sanlibaba, 2020; Liu et al., 2017; Massawe et al., 2019; Pereira et al., 2018) .

To the best of our knowledge, this is the first study in Portugal to report MDR among *S. aureus* isolated from Portuguese RTE and non-RTE sausages. Although the MDR rate observed was overall low, the presence of MDR strains poses a significant public health risk and highlights the need for continued surveillance and intervention to prevent the emergence and spread of novel resistance traits.

5.3 Principal component analysis of antibiotic resistance

As a first step, a correlation matrix was generated based on antimicrobial susceptibility results to evaluate any potential associations between resistance to different antimicrobial classes among *S. aureus* isolates. Table 4 presents the correlation coefficients among pair-wise resistances to eight antibiotics. The values close to 1 indicate strong positive correlations, while the values close to -1 indicate strong negative correlations. The analysis revealed several notable patterns of association (Table 4), described as follows:

Table 4: Correlation analysis of *Staphylococcus aureus* resistance to antibiotics

Antibiotic	**PEN**	**NOR**	**GEN**	**TOB**	**ERY**	**CLI**	**TCY**	**TMP**
PEN	1.000							
NOR	-0.484	1.000						
GEN	0.346	-0.139	1.000					
TOB	0.513	-0.207	**0.674**	1.000				
ERY	0.140	-0.376	0.464	**0.689**	1.000			
CLI	-0.051	-0.194	-0.127	-0.188	-0.298	1.000		
TCY	0.387	-0.169	**0.983**	**0.645**	0.439	-0.074	1.000	
TMP	0.346	-0.139	**1.000**	**0.674**	0.464	-0.127	**0.984**	1.000

Penicillin (PEN); norfloxacin (NOR); gentamycin (GEN); tobramycin (TOB); erythromycin (ERY); clindamycin (CLI); tetracycline (TCY); trimethoprim (TMP).

A very Strong positive correlation was shown between TCY and GEN (r=0.983), also between TMP and both GEN and TCY (r=1.000, r=0.984) respectively, hypothesizing possible co-resistance or linked mechanisms between antibiotics. While TOB shows a moderate to approximately strong positive correlation with ERY (r=0.689), with TCY (r=0.645), and a similar correlation between GEN and TMP (r=0.674); a moderate negative correlation was observed between PEN and NOR (r=-0.484). The negative correlation between resistance to NOR and PEN suggests that resistant strains to one are often sensitive to the other. This may reflect collateral sensitivity, an evolutionary trade-off in the development of antimicrobial resistance (Yekani et al., 2023).

Collateral sensitivity may explain this pattern: mutations that confer resistance to NOR (e.g., in gyrA) can at the same time increase susceptibility to other antibiotic classes such us PEN, typically due to pleiotropic effects of resistance mutations (Lázár et al., 2014; Malik et al., 2017; Roemhild et al., 2020).

The correlation between resistance to different antibiotic classes it can be either due to the shared mechanisms of action or the presence of linked genetic determinants generally localized on mobile genetic elements.

Multiple studies have explained these associations: the resistance to aminoglycoside AGs group (GEN and TOB) in pathogenic bacteria includes aminoglycoside-modifying enzymes (AMEs), mutations and modifications of ribosomal targets, and efflux pumps (Douglas et al., 2022; Huang et al., 2018). The predominant mechanism of resistance to AGs antibiotics in *S. aureus* is by the acquisition of cytoplasmic AMEs that modify AGs antibiotic molecules. AGs resistance is dependent on the acquisition of AMEs, including *aac(6')-Ie+aph(2"), ant(4')-Ia, aph(3')-IIIa*, and *ant(6)-Ia* genes, which inactivate the antibiotic. The *aacA-aphD* gene is frequently located on plasmids, which can be transferred between bacteria cells, facilitating the spread of resistance (Loss et al., 2019; Rahimi, 2016).

While the TCY resistance occurs via two principal mechanisms, the initial mechanism involves the active expulsion of the antibiotic via the plasmid-mediated TetK and TetK major facilitator superfamily (MFS) efflux pumps (Zhang et al., 2023). TetK is usually located on a plasmid within the MRSA strain SCCmecIII. The second mechanism involves the expression of TetO and TetM, two ribosome-protecting proteins (RPPs) that bind to the ribosome and obstruct TET from accessing its targets. The *tet(M)* gene is located inside the chromosomes of numerous *S. aureus* strains (Shao et al., 2025).

Resistance to TMP in *S. aureus* isolates can be achieved with a single point mutation, F98Y, in the chromosomal *dfrB* gene that encodes the DHFR enzyme, which is required for DNA synthesis, involve obtaining genes that encode DHFR enzymes that are not easily repressed, so enabling the bypass of the chromosomal DHFR blockage (Hampele et al., 1997; Shao et al., 2025).

The strongly positive correlation between those three antibiotics explains the co-occurrence of resistance among these antibiotic classes, which may be explained by the presence of the genes responsible for these resistance mechanisms that are located on the same mobile genetic element (plasmids or transposons). This genetic association means that when a bacterium acquires resistance to one antibiotic, it may simultaneously gain resistance to others, even if the mechanisms are unrelated. Multiple studies revealed a high resistance association between those antibiotics in *S. aureus*.

A study conducted in Pakistan on raw cow milk reported a strong positive correlation between the presence of resistance genes and phenotypic resistance to GEN, TCY, and TMP, with high rates of relevant genes; 72.34% isolates carried *tetK* gene, and 23.40% carried *aacA-aphD* gene. (Haq et al., 2024). Similarly, a study investigating *S. aureus* from clinical specimens reported that MDR involving TCY, GEN, TMP, and ERY was common, with resistance genes detected in varying proportions, the co-existence of these genes within the same isolates provides a molecular explanation for the positive correlations between resistance to these antibiotics (Brdová et al., 2024). In agreement, research carried out in Durban found that 50–75% of *S. aureus* from broiler chickens were resistant to TMP, TCY, and ERY, suggesting linked resistance due to co-selection and gene transfer (Mkize et al., 2017). The genetic background revealed in these studies supports the frequent co-resistance observed phenotypically, as resistance determinants are often located on mobile genetic elements, facilitating their simultaneous acquisition and expression.

The negative correlation observed between NOR and PEN is explained by the distinct mechanisms of action and resistance between those two classes. PEN resistance arises from the acquisition of the ability to produce β-lactamase/penicillinase, which hydrolyses the β-lactam ring (Brdová et al., 2024; Foster, 2017).

While the resistance to fluoroquinolones (NOR) in *S. aureus* can be caused by several mechanisms: chromosomal mutations, plasmid-acquired resistance genes, and efflux, the most prevalent mechanism of resistance involves mutations in the quinolone-resistance determining regions (QRDRs) of the *gyrA* and *parC* genes, leading to amino acid substitutions (Huynh et al., 2023). The unrelated mechanisms of action and resistance give a better understanding of the negative correlation between those two classes.

5.3.1 Correlation between antibiotic patterns and dimensions

A correlation graph between variables and dimension was constructed to identify which variables contribute most to each dimension (Fig. 12). Correlation coefficients, ranging from -1.0 to 1.0, are visually represented using a colour gradient from dark red (strong negative correlation) to dark blue (strong positive correlation). In addition, the size of each circular marker reflects the strength of the correlation, with larger circles indicating stronger associations.

In dimension 1, the occurrence of GEN resistance was very related with the resistance to TMP and TCY, TOB and ERY, suggesting that these resistance traits frequently co-occur in the same isolates, possibly due to shared genetic determinants or selective pressures (Haq et al., 2024; Lika et al., 2021). While NOR resistance was the highest variable in dimension 2 and which was correlated with PEN and CLI. In Dimension 3, CLI was the most important variable and was moderately correlated with ERY.

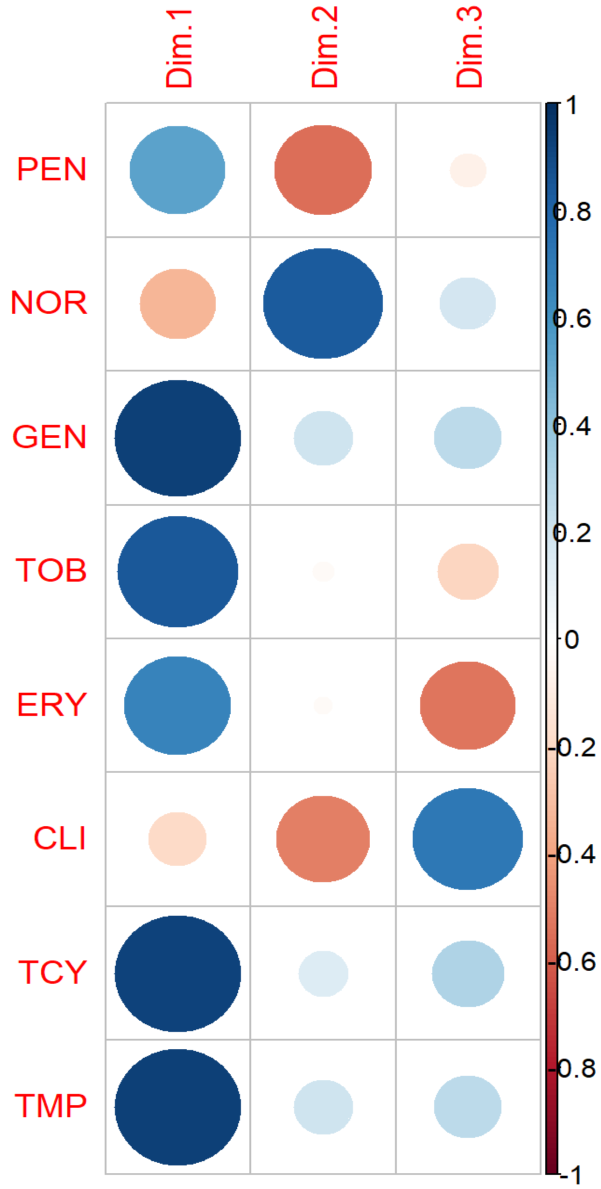
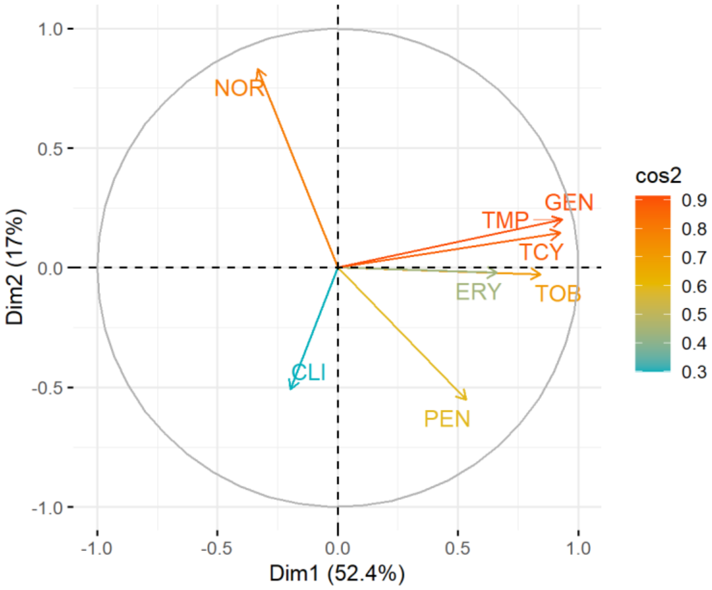


Figure 12: Correlation of resistant antibiotics vs dimension. Dim 1: Dimension 1. Dim 2: Dimension 2. Dim 3: Dimension 3

Variables: penicillin (PEN); norfloxacin (NOR); gentamycin (GEN); tobramycin (TOB); erythromycin (ERY); clindamycin (CLI); tetracycline (TCY); trimethoprim (TMP).

5.3.1 Correlation of antibiotic patterns

Figure 13 represent through bi-dimensional maps the results presented in Table 5 of antibiotic resistance variables, based on the solution of the three-component PCA. Such visualizations provide a better understanding of potential associations and clustering patterns between different antibiotics, highlighting how certain resistance traits may be linked between components.



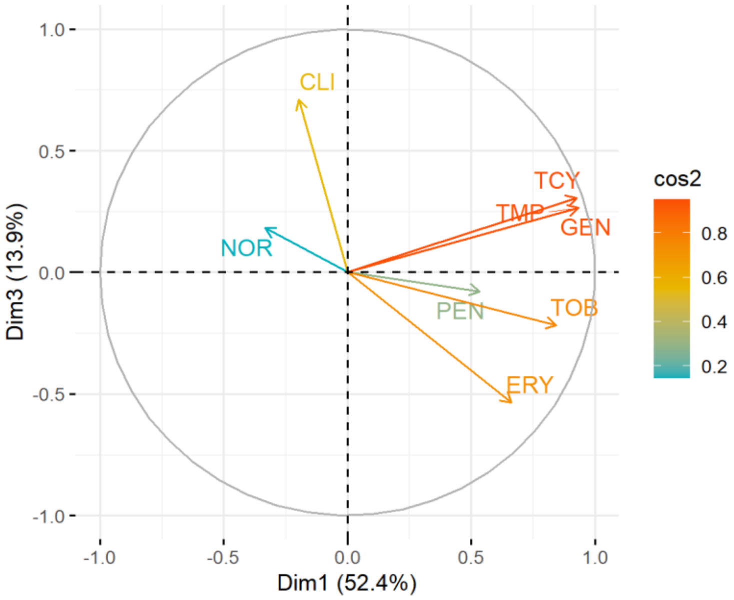


Figure 13: Antibiotic loadings on the three-component resistance map of *Staphylococcus aureus* isolated from RTE and non-RTE Portuguese sausages.

Penicillin (PEN); norfloxacin (NOR); gentamycin (GEN); tobramycin (TOB); erythromycin (ERY); clindamycin (CLI); tetracycline (TCY); trimethoprim (TMP).

Table 5: Coefficients of correlation of antibiotic resistance variables with the three principal components (Dim.1, Dim.2, and Dim.3), along with explained variances and cumulative variances

Variable	Dim.1	Dim.2	Dim.3
PEN	0.5341	-0.5523	-0.0775
NOR	-0.3326	0.8321	0.1816
GEN	0.9339	0.2009	0.2655
TOB	0.8445	-0.0276	-0.2170
ERY	0.6631	-0.0206	-0.5351
CLI	-0.1960	-0.5095	0.7102
TCY	0.9255	0.1445	0.3060
TMP	0.9339	0.2009	0.2655
Variance	4.188	1.360	1.111
% of variance	**52.355**	**16.998**	**13.892**
Cumulative % of variance	52.355	69.353	83.245

(Dim1) Dimension 1, (Dim2) Dimension 2, (Dim3) Dimension 3. penicillin (PEN); norfloxacin (NOR); gentamycin (GEN); tobramycin (TOB); erythromycin (ERY); clindamycin (CLI); tetracycline (TCY); trimethoprim (TMP).

Dim1, the first and most important component, accounted for 52.4% of the variance registered. It was strongly positively correlated to resistance to GEN, TMP, and TCY, and moderately to TOB, and less with ERY. Still, all these antibiotics were closely associated, suggesting the possibility of an underlying shared resistance mechanism, either through MDR mechanisms, efflux pump systems, target site modifications, or enzymatic modifications. PEN also shows a positive correlation with Dim1, although its contribution is moderate. This may reflect partial overlap in resistance mechanisms, such as β-lactamase-producing strains that also harbour other resistance genes.

The second component, Dim2, justified 17% of the data and was highly but negatively correlated with PEN and CLI, and strongly positively correlated with NOR. It was also observed that the NOR resistance was independent of the resistance to TOB, GEN, ERY, TCY and TMP.

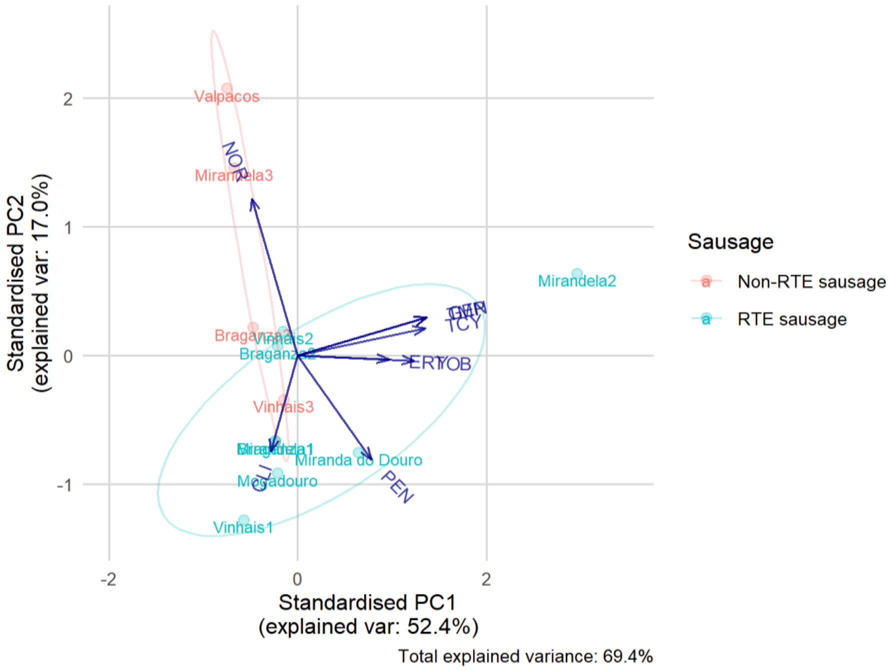
As for the third component, Dim3 explained a marginal 14% of the data variance and was highly positively correlated with CLI and moderately associated with NOR resistance, while showing a negative correlation with ERY resistance. This pattern is logical since no inducible or constitutive CLI resistance (iMLSb/ cMLSb) was detected in our isolates. Although both are protein synthesis inhibitors, the observed divergence suggests that resistance to CLI in our population is independent of or even inversely related to ERY resistance (Miklasińska-Majdanik, 2021).

In contrast with previous studies, Lin et. al. (2019) reported a higher constitutive resistance (ERY=46.3%, CLI=40.7%) than inducible resistance (18.5%), while Prabhu et. al. (2011) reported that 37.52% of isolates tested positive for inducible CLI resistance by D-test, whereas the remaining isolates were negative for D-test, out of which 18 (16.66%) were shown to have constitutive CLI resistance, and 29.62% showed true sensitivity to CLI (MS phenotype) (Lin et al., 2019; Prabhu et al., 2011).

As far as we are aware, no prior finding in Portugal has reported zero prevalence of both inducible and constitutive CLI resistance in *Staphylococcus aureus* strains isolated from meat products.

5.3.2 Producer’s location influence on phenotypic antibiotic resistance of *Staphylococcus aureus* in RTE and non-RTE sausages

Figure 14 represents a visualization of multivariate relationships between the distribution of *S. aureus* isolates recovered from the RTE and non-RTE sausages produced in different regions and their resistance profiles to various antibiotics. Each point represents the centroid or samples’ average from a specific region; the colour represents sausage type; and the grouping or separation of these points provides insight into how resistance patterns vary by location.



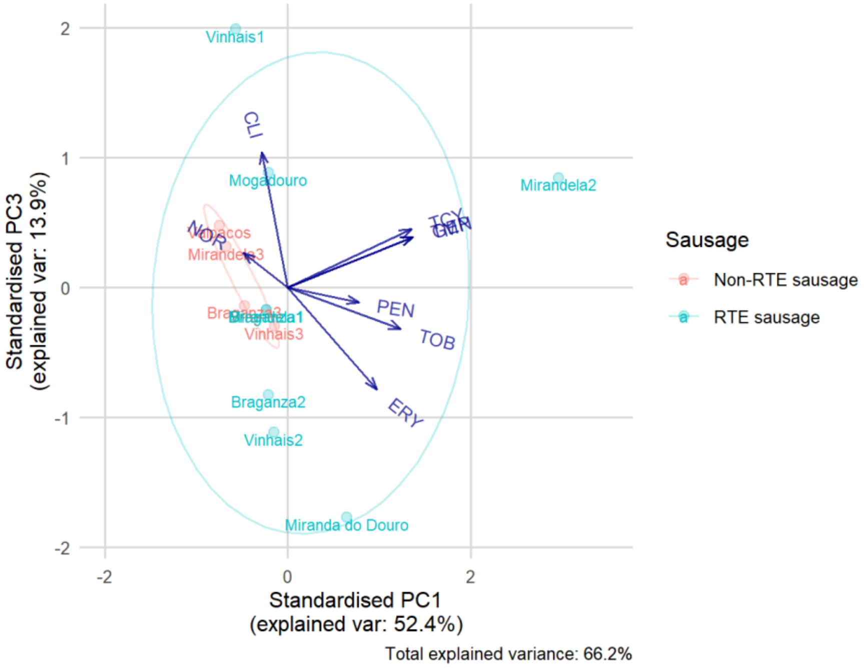


Figure 14: Producer's location centroids projected on the three-component antibiotic resistance map of *Staphylococcus aureus* isolated from RTE and non-RTE sausages.

Penicillin (PEN); norfloxacin (NOR); gentamycin (GEN); tobramycin (TOB); erythromycin (ERY); clindamycin (CLI); tetracycline (TCY); trimethoprim (TMP).

Figure 14 (top) illustrates that the first principal component (Dim1), explaining 52.4% of total variance, distinctly categorizes most RTE sausage producers (light blue) to the right. This suggests that these producers are strongly associated with high resistance to TCY, TMP, GEN, TOB, PEN and a lesser extent ERY. The close grouping of these antibiotic vectors suggests the probability of co-resistance mechanisms, particularly evident in Mirandela samples. In contrast, the non-RTE sausage producers (in light red), particularly samples from Valpaços and Mirandela3, more associated with Dimension 2, are more aligned with the second component (Dim2; 17.0%) and associated with (NOR) resistance, indicating a distinct resistance profile.

Figure 14 (bottom) highlights the correlation of CLI, which contributes significantly to the third component (13.9% of variance) and is more closely associated with the Mogadouro and Vinhais samples, suggesting unique variability in CLI resistance in these areas. NOR, on the other hand, remains most closely associated with the Alheira de Mirandela and Valpaços samples, with a lower contribution.

Even though principal component analysis reveals certain patterns, such as the tendency of RTE sausage producers to be associated with co-resistance to several antibiotics, and that of certain non-RTE sausage producers to be associated with resistance to NOR, these tendencies do not correspond to strict regional differentiation. The resistance profiles observed are found in all regions and show no specific association with any particular location, indicating heterogeneity in resistance mechanisms both between types of products and between producer’s locations.

VI. CONCLUSION

The present investigation is the first report describing the phenotypic antimicrobial resistance profile of *Staphylococcus aureus* isolated from Portuguese fermented meat products chouriça (ready-to-eat) and alheira (not ready-to-eat) produced in northern Portugal. Out of the 47 isolates tested, 68% showed resistance to at least one of the 14 antimicrobials tested. PEN and ERY emerged as the most widespread resistance phenotypes, while resistance to TCY was infrequent, and resistance to NOR was detected exclusively in non-ready-to-eat food isolates. It is important to note that no methicillin-resistant *S. aureus* (MRSA) strains were detected, and multidrug resistance (MDR), defined as resistance to three or more classes of antibiotics, was observed in only 6.4% of isolates.

Correlation and principal component analyses (PCA) provided deeper insight into the structure of resistance patterns. Strong positive associations between TCY, TMP, GEN, TOB, and ERY suggest the presence of co-selection mechanisms or linked genetic determinants, possibly mediated by mobile genetic elements. In contrast, a moderate negative correlation between PEN and NOR resistance highlights a potential case of collateral sensitivity, reflecting an evolutionary trade-off whereby resistance to one antimicrobial may increase susceptibility to another.

Dimensional analysis also revealed product-specific associations, with isolates from ready-to-eat sausages linked primarily to resistance to β-lactams and protein synthesis inhibitors (ERY, TCY, GEN, CLI), while isolates from non-ready-to-eat products were more closely associated with resistance to fluoroquinolones (NOR). However, these trends did not reflect a strict geographical clustering. Instead, resistance traits were heterogeneously distributed across production regions, likely reflecting the impact of artisanal processing practices, variability in raw material quality, and differences in antimicrobial exposure throughout the food chain.

These findings clearly represent a significant public health concern. The detection of clinically relevant antimicrobial resistance, including β-lactams, macrolides, aminoglycosides, and fluoroquinolones, in *S. aureus* isolated from traditional fermented sausages highlights the potential role of these products as reservoirs of antimicrobial resistance (AMR) determinants. Despite the low prevalence of MDR in our study, the co-resistance patterns observed highlight the risk of horizontal gene transfer and the likelihood of resistance expansion under selective pressure.

Considering the cultural and economic relevance of traditional Portuguese sausages, it is essential to enhance hygiene practices during processing, implement stricter surveillance and control measures, and strengthen national AMR surveillance programmes to mitigate the spread of resistant strains in the food chain.

VII. FUTURE PERSPECTIVES

This study holds considerable importance as it addresses the major concern of antimicrobial resistance (AMR) in foodborne *Staphylococcus aureus* isolates from traditional Portuguese sausages, a relevant reservoir for resistant bacteria that may cause foodborne outbreaks. Understanding resistance profiles in these artisanal food products is crucial for mapping the pathways through which resistant strains can enter the human population via the food chain. Considering the growing global threat of AMR, particularly in pathogenic bacteria that compromise treatment options, studies such as this provide valuable baseline data necessary for the implementation of effective surveillance and intervention strategies.

Future research should aim to broaden the investigation by including a larger variety of traditional meat products and extending sampling to several geographical regions of Portugal, to provide a more comprehensive overview of antimicrobial resistance (AMR) in foodborne pathogens at the national level. The combination of advanced molecular approaches, especially whole genome sequencing (WGS), with the phenotypic analysis, would allow for a more detailed characterization of resistance mechanisms, horizontal gene transfer events, and clonal relationships between isolates. This genotypic evidence is essential for the early detection of emerging resistant strains and for the design of targeted, evidence-based control strategies that mitigate the spread of AMR in the food chain.

Longitudinal studies investigating temporal trends in AMR are essential for evaluating the long-term impact of policy interventions, such as legislation governing antimicrobial use in livestock farming and food processing environments. These studies would provide valuable evidence on the effectiveness of current strategies and inform the design of more adaptive policies. Furthermore, combining predictive microbiology and quantitative modelling in future research is a promising approach for advancing food safety assessment.

By modelling, survival, and development of resistance in *Staphylococcus aureus* under various environmental and processing conditions, these predictive tools can generate risk scenarios that estimate the likelihood of resistance emerging. This approach not only improves risk assessment but also provides a scientific basis for risk management decisions, ultimately contributing to safer production of traditional meat products.

Alternative antimicrobial strategies, such as bacteriophages, antimicrobial peptides, and biocontrol agents like lactic acid bacteria (LAB), show great promise for sustainably mitigating AMR in traditional food production systems while preserving product authenticity. LAB are recognized for their potential to inhibit foodborne pathogens and produce bioactive compounds, offering a natural, safe, and effective means of bio-preservation.

The complexity of AMR spread cannot be addressed simply by one sector alone, as it involves interactions between humans, animals, food, and the environment. Adopting a One Health framework is therefore essential, as it integrates knowledge from public health, veterinary medicine, microbiology, and food safety. Strengthening cross-sector collaboration will improve surveillance networks and facilitate the development of comprehensive strategies to reduce the burden of AMR from food sources. Pursuing research and cooperation in these areas is essential to protect public health, maintain the effectiveness of antimicrobial agents, and support sustainable food production practices.

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IX. ANNEX

Annex 1 : Disk diffusion data set for *Staphylococcus aureus* isolates

Food Source	Location County	Bacteria	Ist Code	Rpt	Mean (PEN)	SD	PEN	Mean (FOX)	SD	S/R	Mean (NOR)	SD	S/R	Mean (AMK)	SD	S/R	Mean (GEN)	SD	S/R
Chourica	Vinhais	*S. aureus*	DR1	3	32,13	0,14	S	29,54	0,58	S	26,10	0,42	S	23,01	0,36	S	23,94	0,33	S
Chourica	Vinhais	*S. aureus*	DR2	3	13,52	0,33	R	31,77	0,17	S	27,32	0,02	S	23,01	0,19	S	25,83	0,09	S
Chourica	Vinhais	*S. aureus*	DR4	3	31,31	0,67	S	29,87	0,26	S	27,33	0,09	S	22,13	0,12	S	24,00	0,37	S
Chourica	Mogadouro	*S. aureus*	BP3	3	31,64	0,08	S	30,76	0,29	S	28,21	0,09	S	23,02	0,14	S	26,31	0,09	S
Chourica	Mogadouro	*S. aureus*	BP4	3	14,98	0,31	R	31,85	0,15	S	28,30	0,05	S	23,72	0,15	S	25,77	0,14	S
Chourica	Mogadouro	*Staph spp.*	BP2.1	3	32,96	0,64	S	31,86	0,14	S	31,28	0,51	S	27,30	0,12	S	29,91	0,11	S
Chourica	Mogadouro	*Staph spp.*	BP1.1	3	17,32	0,14	R	34,77	0,21	S	31,56	0,48	S	25,60	0,04	S	29,72	0,15	S
Chourica	Miranda do Douro	*Staph spp.*	PDO1	3	15,34	0,54	R	32,84	0,10	S	34,12	0,46	S	26,74	0,17	S	29,61	0,11	S
Chourica	Mirandela	*Staph spp.*	FV3	3	19,12	0,31	R	32,68	0,09	S	30,06	0,14	S	27,91	0,13	S	30,09	0,43	S
Chourica	Mirandela	*Staph spp.*	FV2	3	16,55	0,45	R	31,99	0,33	S	34,26	0,07	S	24,64	0,04	S	24,98	0,20	S
Chourica	Mogadouro	*S. aureus*	BP1.2	3	12,76	0,17	R	28,83	0,05	S	26,21	0,15	S	23,67	0,14	S	25,14	0,27	S
Chourica	Mirandela	*S. aureus*	ML1	3	10,69	0,79	R	28,91	0,25	S	26,07	0,12	S	21,09	0,07	S	11,95	0,14	R
Chourica	Bragança	*S. aureus*	SF4	3	13,11	0,06	R	28,78	0,18	S	27,80	0,10	S	24,80	0,33	S	27,06	0,17	S
Chourica	Mogadouro	*S. aureus*	BP5	3	14,98	0,31	R	31,85	0,15	S	28,30	0,05	S	23,72	0,15	S	25,77	0,14	S
Chourica	Bragança	*S. aureus*	PG3ST1	3	30,13	0,26	S	32,06	0,22	S	30,13	0,20	S	23,94	0,23	S	24,93	0,09	S

Chourica	Bragança	*S. aureus*	PG4ST1	3	13,33	0,44	R	28,83	0,04	S	27,31	0,03	S	21,59	0,06	S	23,58	0,06	S
Chourica	Bragança	*S. aureus*	PG4ST2	3	31,08	0,11	S	32,09	0,31	S	30,26	0,05	S	24,13	0,25	S	25,56	0,04	S
Chourica	Bragança	*S. aureus*	PG4ST3	3	29,51	0,19	S	28,92	0,17	S	30,25	0,11	S	25,16	0,13	S	27,84	0,44	S
Chourica	Bragança	*S. aureus*	PG4ST4	3	31,67	0,08	S	31,92	0,28	S	31,96	0,14	S	25,88	0,14	S	27,85	0,39	S
Chourica	Bragança	*S. aureus*	PG5ST1	3	29,93	0,29	S	30,99	0,26	S	28,67	0,47	S	26,14	0,04	S	27,20	0,17	S
Chourica	Bragança	*S. aureus*	PG1ST1	3	31,74	0,10	S	30,05	0,34	S	30,30	0,49	S	22,63	0,05	S	23,61	0,15	S
Chourica	Vinhais	*S. aureus*	AC5	3	35,15	0,62	S	33,13	0,15	S	30,87	0,06	S	25,04	0,08	S	27,02	0,28	S
Alheira	Vinhais	*S. aureus*	V1	3	13,92	0,24	R	25,93	0,26	S	26,25	0,21	S	22,40	0,45	S	24,13	0,09	S
Alheira	Vinhais	*S. aureus*	V2	3	7,26	0,19	R	28,10	0,16	S	18,32	0,30	S	21,13	0,11	S	20,17	0,14	S
Alheira	Vinhais	*S. aureus*	V3	3	7,55	0,49	R	27,10	0,11	S	16,08	0,68	R	20,57	0,38	S	20,24	0,09	S
Alheira	Valpacos	*S. aureus*	SM4	3	29,84	0,02	S	25,62	0,06	S	13,80	0,09	R	20,43	0,59	S	19,26	0,10	S
Alheira	Bragança	*S. aureus*	B1	3	30,84	0,05	S	29,95	0,11	S	29,88	0,15	S	22,90	0,29	S	23,87	0,25	S
Alheira	Mirandela	*S. aureus*	FN3(A)	3	30,15	0,09	S	25,94	0,16	S	25,88	0,01	S	22,00	0,10	S	21,18	0,11	S
Alheira	Mirandela	*S. aureus*	FN4(A)	3	29,71	0,12	S	26,13	0,13	S	14,58	0,83	R	21,08	0,07	S	20,91	0,36	S
Alheira	Mirandela	*S. aureus*	FN5(A)	3	30,28	0,09	S	25,87	0,20	S	14,58	0,87	R	21,01	0,00	S	21,01	0,11	S
Alheira	Bragança	*S. aureus*	B6	3	27,55	0,62	S	27,74	0,07	S	27,66	0,05	S	24,49	0,49	S	24,96	0,05	S
Alheira	Bragança	*S. aureus*	B7	3	28,57	0,42	S	27,75	0,07	S	27,83	0,11	S	24,69	0,10	S	25,71	0,22	S
alheira	Vinhais	*S. aureus*	AV2-C	3	25,03	0,15	S	27,30	0,12	S	28,26	0,11	S	25,08	0,25	S	26,18	0,02	S
Alheira	Vinhais	*S. aureus*	AV2-1	3	23,55	0,76	R	25,21	0,08	S	27,42	0,38	S	22,09	0,11	S	23,07	0,06	S
Alheira	Vinhais	*S. aureus*	AV2-4	3	15,98	0,23	R	29,04	0,32	S	28,88	0,16	S	23,14	0,08	S	23,82	0,01	S
Alheira	Vinhais	*Staph spp.*	AV2-11	3	37,25	0,13	S	33,26	0,31	S	30,93	0,06	S	26,60	0,05	S	26,93	0,23	S
Alheira	Vinhais	*S. aureus*	AV3-2D7	3	12,39	0,09	R	28,00	0,20	S	27,77	0,06	S	25,13	0,07	S	25,72	0,15	S

Alheira	Vinhais	*S. aureus*	AV2-4D7	3	15,09	0,07	R	30,98	0,34	S	31,02	0,40	S	24,88	0,46	S	25,38	0,05	S
Alheira	Vinhais	*S. aureus*	AV3-1	3	14,78	0,11	R	29,80	0,03	S	19,18	0,24	S	27,68	0,08	S	28,06	0,03	S
Alheira	Vinhais	*S. aureus*	AV3-2D2	3	15,85	0,26	R	31,23	0,19	S	32,37	0,15	S	23,95	0,13	S	25,77	0,21	S
Alheira	Vinhais	*S. aureus*	AV3-3	3	15,21	0,09	R	34,06	0,33	S	34,22	0,90	S	23,84	0,18	S	24,38	0,08	S
alheira	Bragança	*S. aureus*	AB3-C	3	27,96	0,28	S	28,24	0,04	S	31,09	0,49	S	24,82	0,30	S	24,04	0,05	S
Alheira	Bragança	*S. aureus*	AB3-T	3	32,12	0,19	S	30,30	0,84	S	28,83	1,19	S	23,33	0,05	S	24,34	0,05	S
Alheira	Bragança	*S. aureus*	AB3-1	3	29,61	0,13	S	27,77	0,13	S	29,84	0,33	S	25,69	0,07	S	24,82	0,12	S
Alheira	Bragança	*S. aureus*	AB3-3	3	28,95	0,05	S	26,96	0,20	S	23,15	0,19	S	25,11	0,09	S	22,83	0,12	S
Alheira	Bragança	*S. aureus*	AB3-11	3	33,92	0,20	S	33,86	0,20	S	30,55	0,04	S	25,00	0,16	S	20,67	0,13	S
Alheira	Bragança	*S. aureus*	AB3-2	3	33,95	0,39	S	31,32	0,68	S	30,00	0,25	S	26,07	0,10	S	21,06	0,54	S

Food Source	**Location County**	**Bacteria**	**Ist code**	**Rpt**	**Mean (TOB)**	**SD**	**S/R**	**Mean (ERY)**	**SD**	**S/R**	**Mean (CLI)**	**SD**	**S/R**	**Mean (TCY)**	**SD**	**S/R**	**Mean (LNZ)**	**SD**	**S/R**
Chourica	Vinhais	*S. aureus*	DR1	3	23,95	0,34	S	25,87	0,26	S	7,54	0,57	R	29,70	0,09	S	27,76	0,20	S
Chourica	Vinhais	*S. aureus*	DR2	3	26,67	0,15	S	27,01	0,26	S	27,81	0,10	S	28,94	0,28	S	28,58	0,03	S
Chourica	Vinhais	*S. aureus*	DR4	3	24,79	0,16	S	26,69	0,18	S	25,81	0,07	S	27,92	0,45	S	26,93	0,24	S
Chourica	Mogadouro	*S. aureus*	BP3	3	25,26	0,11	S	26,15	0,04	S	19,02	0,09	R	15,25	0,16	R	27,89	0,14	S
Chourica	Mogadouro	*S. aureus*	BP4	3	25,80	0,17	S	28,08	0,25	S	27,88	0,25	S	30,90	0,19	S	29,17	0,01	S
Chourica	Mogadouro	*Staph spp.*	BP2.1	3	28,10	0,26	S	28,53	0,28	S	29,32	0,62	S	33,82	0,16	S	30,05	0,21	S
Chourica	Mogadouro	*Staph spp.*	BP1.1	3	29,45	0,16	S	9,25	0,19	R	31,29	0,21	S	32,83	0,13	S	30,58	0,45	S
Chourica	Miranda do Douro	*Staph spp.*	PDO1	3	16,75	0,03	S	8,97	0,12	R	28,83	0,06	S	31,17	0,23	S	30,76	0,20	S
Chourica	Mirandela	*Staph spp.*	FV3	3	30,80	0,13	S	30,87	0,34	S	30,83	0,17	S	28,84	0,34	S	29,70	0,11	S
Chourica	Mirandela	*Staph spp.*	FV2	3	25,94	0,33	S	28,79	0,39	S	27,73	0,16	S	32,94	0,04	S	26,29	0,27	S
Chourica	Mogadouro	*S. aureus*	BP1.2	3	25,23	0,11	S	27,00	0,29	S	26,92	0,28	S	28,81	0,04	S	30,94	0,12	S
Chourica	Mirandela	*S. aureus*	ML1	3	13,79	0,18	S	8,33	0,04	R	25,01	0,26	S	10,78	0,40	R	29,17	0,01	S
Chourica	Braganza	*S. aureus*	SF4	3	26,68	0,14	S	28,97	0,66	S	28,34	0,45	S	28,08	0,16	S	29,16	0,15	S
Chourica	Mogadouro	*S. aureus*	BP5	3	25,80	0,17	S	28,08	0,25	S	27,88	0,25	S	30,90	0,19	S	28,56	0,06	S
Chourica	Bragança	*S. aureus*	PG3ST1	3	25,31	0,12	S	0,00	0,00	R	28,55	0,39	S	28,63	0,39	S	29,79	0,30	S
Chourica	Bragança	*S. aureus*	PG4ST1	3	23,60	0,05	S	27,24	0,08	S	26,12	0,17	S	28,68	0,13	S	31,02	0,07	S
Chourica	Bragança	*S. aureus*	PG4ST2	3	25,16	0,06	S	0,00	0,00	R	28,82	0,15	S	30,05	0,07	S	30,95	0,33	S

Chourica	Bragança	*S. aureus*	PG4ST3	3	28,08	0,05	S	0,00	0,00	R	26,61	0,04	S	30,97	0,34	S	30,99	0,30	S
Chourica	Bragança	*S. aureus*	PG4ST4	3	27,18	0,09	S	0,00	0,00	R	28,05	0,27	S	29,88	0,27	S	30,55	0,02	S
Chourica	Bragança	*S. aureus*	PG5ST1	3	28,70	0,13	S	0,00	0,00	R	28,25	0,10	S	28,86	0,36	S	30,04	0,15	S
Chourica	Bragança	*S. aureus*	PG1ST1	3	23,81	0,04	S	29,03	0,04	S	29,17	0,11	S	29,84	0,13	S	28,12	0,15	S
Chourica	Vinhais	*S. aureus*	AC5	3	26,88	0,29	S	0,00	0,00	R	32,06	0,32	S	30,75	0,20	S	25,76	0,16	S
Alheira	Vinhais	*S. aureus*	V1	3	24,69	0,14	S	28,33	0,44	S	27,89	0,11	S	28,08	0,18	S	26,64	0,37	S
Alheira	Vinhais	*S. aureus*	V2	3	21,72	0,13	S	25,99	0,20	S	25,07	0,49	S	25,83	0,25	S	25,83	0,34	S
Alheira	Vinhais	*S. aureus*	V3	3	21,79	0,22	S	26,57	0,04	S	26,82	0,31	S	25,67	0,12	S	30,73	0,10	S
Alheira	Valpacos	*S. aureus*	SM4	3	20,11	0,22	S	25,22	0,07	S	25,89	0,12	S	25,72	0,23	S	25,89	0,29	S
Alheira	Bragança	*S. aureus*	B1	3	24,06	0,12	S	8,11	0,08	R	29,82	0,27	S	30,60	0,46	S	25,95	0,27	S
Alheira	Mirandela	*S. aureus*	FN3(A)	3	22,01	0,14	S	26,30	0,02	S	27,18	0,11	S	27,13	0,01	S	26,68	0,04	S
Alheira	Mirandela	*S. aureus*	FN4(A)	3	20,77	0,25	S	25,81	0,08	S	25,87	0,13	S	25,74	0,14	S	28,11	0,14	S
Alheira	Mirandela	*S. aureus*	FN5(A)	3	21,84	0,09	S	26,09	0,37	S	26,92	0,18	S	25,95	0,35	S	30,31	0,03	S
Alheira	Bragança	*S. aureus*	B6	3	26,82	0,10	S	29,61	0,15	S	29,70	0,05	S	27,66	0,13	S	29,86	0,29	S
Alheira	Bragança	*S. aureus*	B7	3	24,96	0,31	S	30,05	0,07	S	30,00	0,20	S	28,11	0,14	S	26,03	0,26	S
alheira	Vinhais	*S. aureus*	AV2-C	3	25,87	0,05	S	28,72	0,16	S	28,66	0,16	S	28,87	0,08	S	30,17	0,48	S
Alheira	Vinhais	*S. aureus*	AV2-1	3	22,77	0,07	S	28,97	0,14	S	29,11	0,08	S	25,60	0,06	S	31,72	0,12	S
Alheira	Vinhais	*S. aureus*	AV2-4	3	25,69	0,12	S	28,19	0,10	S	28,84	0,05	S	27,77	0,06	S	28,85	0,25	S
Alheira	Vinhais	*Staph spp.*	AV2-11	3	27,17	0,20	S	30,80	0,23	S	31,04	0,07	S	31,21	0,10	S	32,19	0,23	S
Alheira	Vinhais	*S. aureus*	AV3-2D7	3	22,45	0,10	S	28,13	0,09	S	29,88	0,26	S	26,91	0,34	S	32,71	0,13	S
Alheira	Vinhais	*S. aureus*	AV2-4D7	3	27,35	0,04	S	7,63	0,26	R	30,91	0,34	S	11,28	0,15	R	31,13	0,25	S
Alheira	Vinhais	*S. aureus*	AV3-1	3	23,43	0,10	S	30,84	0,27	S	30,92	0,31	S	30,67	0,09	S	31,68	0,06	S
Alheira	Vinhais	*S. aureus*	AV3-2D2	3	21,87	0,15	S	9,51	0,77	R	30,55	0,04	S	30,15	0,08	S	30,13	0,42	S
Alheira	Vinhais	*S. aureus*	AV3-3	3	21,04	0,15	S	8,65	0,30	R	30,19	0,26	S	28,94	0,24	S	29,73	0,25	S
alheira	Bragança	*S. aureus*	AB3-C	3	20,95	0,15	S	31,13	1,25	S	31,00	0,40	S	27,65	0,11	S	30,08	0,04	S
Alheira	Bragança	*S. aureus*	AB3-T	3	22,58	0,64	S	30,84	0,93	S	30,22	0,15	S	28,92	0,64	S	29,70	0,29	S
Alheira	Bragança	*S. aureus*	AB3-1	3	20,78	0,21	S	29,20	0,08	S	29,32	0,10	S	28,83	0,11	S	30,30	0,11	S
Alheira	Bragança	*S. aureus*	AB3-3	3	17,79	0,06	S	28,96	0,25	S	27,24	0,20	S	27,12	0,09	S	36,09	0,42	S
Alheira	Bragança	*S. aureus*	AB3-11	3	20,96	0,18	S	31,25	0,91	S	30,36	0,72	S	28,87	0,44	S	30.18	30.32	S
Alheira	Bragança	*S. aureus*	AB3-2	3	20,92	0,14	S	32,70	0,47	S	32,02	0,72	S	28,27	0,17	S	36.45	36.2	S

Food_ Source	Location_ County	Bacteria	Ist_ Code	Rpt	Mean (RIF)	SD	RIF	Mean (TMP)	SD	TMP	Mean (SXT)	SD	AST_SXT	Mean (CHL)	SD	CHL
Chourica	Vinhais	*S. aureus*	DR1	3	34,03	0,27	S	29,76	0,13	S	30,23	0,14	S	23,92	0,10	S
Chourica	Vinhais	*S. aureus*	DR2	3	31,92	0,24	S	30,83	0,38	S	31,94	0,30	S	25,32	0,12	S
Chourica	Vinhais	*S. aureus*	DR4	3	33,98	0,36	S	28,77	0,15	S	29,91	0,31	S	25,11	0,31	S
Chourica	Mogadouro	*S. aureus*	BP3	3	41,63	0,09	S	26,71	0,07	S	30,20	0,15	S	24,24	0,15	S
Chourica	Mogadouro	*S. aureus*	BP4	3	34,97	0,08	S	28,29	0,11	S	27,08	0,06	S	27,15	0,13	S
Chourica	Mogadouro	*Staph spp.*	BP2.1	3	39,42	0,53	S	30,75	0,13	S	35,05	0,13	S	26,13	0,05	S
Chourica	Mogadouro	*Staph spp.*	BP1.1	3	36,11	0,29	S	27,77	0,15	S	32,65	0,06	S	27,86	0,16	S
Chourica	Miranda do Douro	*Staph spp.*	PDO1	3	44,67	0,16	S	30,69	0,14	S	27,75	0,17	S	27,75	0,34	S
Chourica	Mirandela	*Staph spp.*	FV3	3	37,13	0,10	S	31,79	0,13	S	27,94	0,22	S	29,12	0,97	S
Chourica	Mirandela	*Staph spp.*	FV2	3	37,20	0,17	S	35,09	0,03	S	32,92	0,03	S	27,77	0,16	S
Chourica	Mogadouro	*S. aureus*	BP1.2	3	33,25	0,23	S	25,93	0,25	S	28,21	0,10	S	27,06	0,22	S
Chourica	Mirandela	*S. aureus*	ML1	3	29,56	0,50	S	0,00	0,00	R	32,19	0,29	S	25,23	0,10	S
Chourica	Braganza	*S. aureus*	SF4	3	33,07	0,55	S	25,54	0,33	S	27,89	0,03	S	26,75	0,13	S
Chourica	Mogadouro	*S. aureus*	BP5	3	34,97	0,08	S	28,29	0,11	S	27,08	0,06	S	27,15	0,13	S
Chourica	Bragança	*S. aureus*	PG3ST1	3	35,58	0,60	S	27,81	0,20	S	32,25	0,17	S	25,52	0,43	S
Chourica	Bragança	*S. aureus*	PG4ST1	3	32,91	0,24	S	28,39	0,24	S	30,71	0,32	S	26,06	0,17	S
Chourica	Bragança	*S. aureus*	PG4ST2	3	35,61	0,37	S	27,11	0,34	S	29,75	0,28	S	26,28	0,10	S
Chourica	Bragança	*S. aureus*	PG4ST3	3	38,13	0,12	S	25,26	0,04	S	32,75	0,10	S	27,90	0,62	S
Chourica	Bragança	*S. aureus*	PG4ST4	3	37,34	0,45	S	26,21	0,11	S	33,02	0,41	S	27,19	0,12	S
Chourica	Bragança	*S. aureus*	PG5ST1	3	37,79	0,18	S	28,69	0,20	S	33,86	0,14	S	27,87	0,21	S
Chourica	Bragança	*S. aureus*	PG1ST1	3	35,18	0,05	S	30,28	0,25	S	31,84	0,11	S	27,16	0,12	S
Chourica	Vinhais	*S. aureus*	AC5	3	37,78	0,22	S	33,17	0,35	S	35,08	0,04	S	27,76	0,10	S
Alheira	Vinhais	*S. aureus*	V1	3	33.66	0,15	S	26,70	0,13	S	26,83	0,27	S	23,82	0,31	S
Alheira	Vinhais	*S. aureus*	V2	3	28.96	0,20	S	21,05	0,16	S	25,10	0,28	S	21,01	0,30	S
Alheira	Vinhais	*S. aureus*	V3	3	28.06	0,22	S	22,14	0,09	S	26,10	0,03	S	23,83	0,28	S
Alheira	Valpacos	*S. aureus*	SM4	3	28.27	0,27	S	26,83	0,26	S	27,68	0,13	S	23,83	0,25	S
Alheira	Braganza	*S. aureus*	B1	3	35.13	0,23	S	30,09	0,11	S	34,39	0,18	S	27,73	0,20	S
Alheira	Mirandela	*S. aureus*	FN3(A)	3	31.91	0,17	S	27,17	0,14	S	29,77	0,15	S	23,88	0,11	S
Alheira	Mirandela	*S. aureus*	FN4(A)	3	30.74	0,07	S	26,70	0,08	S	28,15	0,12	S	24,22	0,11	S
Alheira	Mirandela	*S. aureus*	FN5(A)	3	31.65	0,13	S	26,75	0,36	S	30,55	0,04	S	23,93	0,38	S
Alheira	Bragança	*S. aureus*	B6	3	33.30	0,02	S	30,93	0,51	S	33,78	0,18	S	25,93	0,32	S
Alheira	Bragança	*S. aureus*	B7	3	33.94	0,86	S	28,76	0,17	S	32,17	0,03	S	25,61	0,09	S
alheira	Vinhais	*S. aureus*	AV2-C	3	34.11	0,19	S	32,29	0,04	S	32,76	0,11	S	26,68	0,03	S
Alheira	Vinhais	*S. aureus*	AV2-1	3	31.96	0,47	S	27,26	0,12	S	25,97	0,18	S	23,78	0,07	S
Alheira	Vinhais	*S. aureus*	AV2-4	3	33.37	0,03	S	28,96	0,42	S	31,28	0,20	S	23,09	0,39	S
Alheira	Vinhais	*Staph spp.*	AV2-11	3	40.02	0,20	S	30,69	0,11	S	35,46	0,62	S	29,02	0,05	S
Alheira	Vinhais	*S. aureus*	AV3-2D7	3	33.94	0,25	S	30,95	0,40	S	30,14	0,33	S	25,35	0,11	S
Alheira	Vinhais	*S. aureus*	AV2-4D7	3	35.63	0,35	S	32,73	0,15	S	30,98	0,22	S	28,72	0,11	S
Alheira	Vinhais	*S. aureus*	AV3-1	3	34.14	0,07	S	29,00	0,39	S	31,82	0,18	S	28,94	0,39	S
Alheira	Vinhais	*S. aureus*	AV3-2D2	3	34.75	0,08	S	31,18	0,07	S	31,74	0,14	S	27,22	0,13	S
Alheira	Vinhais	*S. aureus*	AV3-3	3	34.73	0,70	S	30,65	0,07	S	30,99	0,43	S	26,93	0,30	S
alheira	Bragança	*S. aureus*	AB3-C	3	32.53	0,44	S	28,02	0,26	S	30,94	0,30	S	26,71	0,80	S
Alheira	Bragança	*S. aureus*	AB3-T	3	34.86	0,15	S	30,98	0,13	S	31,77	0,09	S	26,96	0,41	S
Alheira	Bragança	*S. aureus*	AB3-1	3	24.56	5,91	S	31,10	0,14	S	34,83	0,15	S	27,46	0,50	S
Alheira	Bragança	*S. aureus*	AB3-3	3	34.19	1,93	S	29,13	0,04	S	36,85	0,51	S	26,95	0,39	S
Alheira	Bragança	*S. aureus*	AB3-11	3	35.08	0,15	S	31,30	0,13	S	35,91	0,05	S	31,57	0,03	S
Alheira	Bragança	*S. aureus*	AB3-2	3	37.68	0,10	S	28,96	0,23	S	36,11	0,14	S	32,92	0,35	S

Annex 2: Representative inhibition patterns of Staphylococcus aureus isolates to various antibiotics.

Fig(A): Disk diffusion showing inhibition zone for Tetracycline (TCY), indicating resistance

Fig(B): Disk Diffusion Assay Demonstrating Gentamycin (GEN) Resistance in *Staphylococcus aureus*

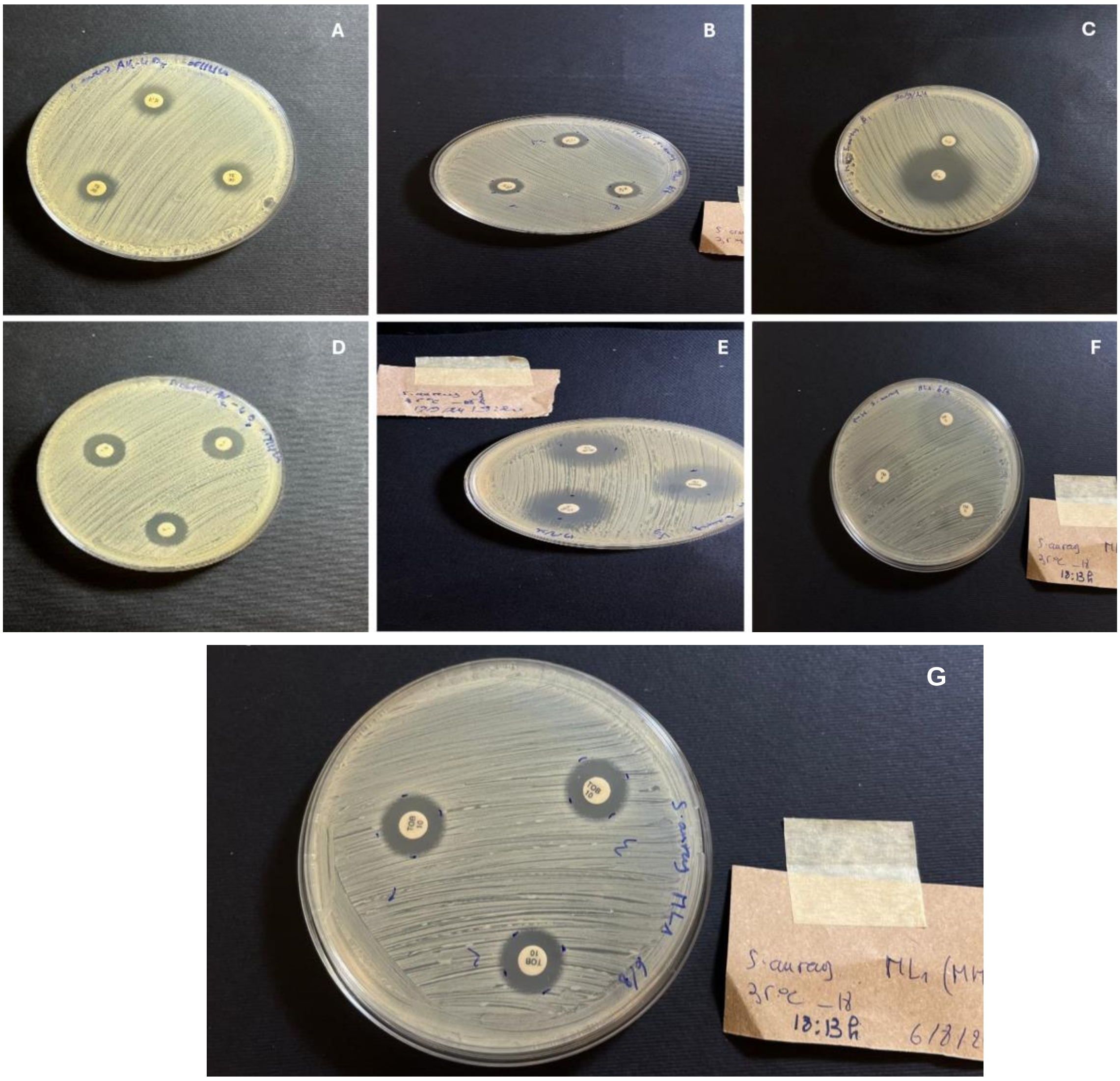
Fig(C): D-Test Assay Revealing Clindamycin (CLI) Susceptibility and Erythromycin (ERY) Resistance in *Staphylococcus aureus*

Fig(D): Representative inhibition zone patterns observed in *S. aureus* isolates showing resistance to β-lactam (PEN) antibiotics.

Fig(E): Disk Diffusion Evidence of Norfloxacin (NOR) Resistance in *S. aureus*

Fig(F): Phenotypic Demonstration of Trimethoprim (TMP) Resistance in *Staphylococcus aureus*

Fig(G): Disk Diffusion Assay Demonstrating Tobramycin (TOB) Resistance in *Staphylococcus aureus*


A
B
C
D
E
F
G
TOB 10
TOB 10
TOB 10
S. aureus ML1 (MH
37°C _18
18:13h 6/8/2