UNIVERSIDADE DE BRASÍLIA

FACULDADE DE AGRONOMIA E MEDICINA VETERINÁRIA
PROGRAMA DE PÓS-GRADUAÇÃO EM SAÚDE ANIMAL

CONCERNS ABOUT ANTIMICROBIAL USE AND PHENOTYPIC RESISTANCE PATTERN IN PIG NASAL MICROBIOME

CONSIDERAÇÕES SOBRE USO DE ANTIMICROBIANOS VIA ORAL EM PORCAS E PERFIL DE RESISTÊNCIA FENOTÍPICA NO MICROBIOMA NASAL DE SUÍNOS.

LUCIANA LANA RIGUEIRA

DOCTORATE THESIS IN ANIMAL HEALTH

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DEDICATORY

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"Plant the seeds of optimism and kindness around you, so that you can reap the fruits of love and happiness".

(Minutes of Wisdom)

Abstract

Title: Concerns about antimicrobial use and resistance pattern in pig nasal microbiome

This study was divided into two parts, both with the aim of evaluating the phenotype of the inhabitants of the pig nasal microbiota. The first study at the University of Brasilia consisted in isolating bacterial agents collected from nasal swabs of 50 sows housed in 10 commercial farms with different sanitary management, located in the Federal District, Brazil. A total of 132 cultured strains were characterized. Microbial susceptibility was evaluated using the qualitative Kirby-Bauer method with up to 23 antibiotic discs, with an overall antimicrobial resistance estimated at 55% (1573/2840 tests). Interestingly, bacitracin, an antimicrobial no longer used in pig production, was found to be the most resistant (92%), followed by florfenicol (76.5%). Molecular diagnosis was performed to confirm the presence of the most common PRDC pathogens. *M. hyopneumoniae* was not detected. *Glaesserella parasuis* and *Actinobacillus pleuropneumoniae* were not cultured but were detected by multiplex polymerase chain reaction (PCR) in 40% and 10% of the farms, respectively. *Pasteurella multocida* was cultured in half of the farms and detected by PCR in 60% of the farms. A positive association was found between the reduction of lesions in the abattoir and the use of autogenous vaccines against *P. multocida* (Fisher's exact probability test, P=0.048). Pathogens such as *Pseudomonas aeruginosa, Actinobacillus suis* and *Salmonella* ser. Typhimurium were the most resistant species, but agents of the commensal nasal microbiota, such as *Staphylococcus* coagulase-negative, also exhibited antimicrobial resistance.

The second study at IRTA-CrESA evaluate antimicrobial susceptibility by broth microdilution on 56 strains obtained from isolates already available in the laboratory, obtained from sow vaginal swab (n=11), colostrum (n=9) and nasal swab samples from dams (n=15) and their litters (n=21). MIC (minimum inhibitory concentration - MIC) values were obtained for 24 antimicrobials. Among the antimicrobials tested, phenotypic resistance to clindamycin was the most common among the strains evaluated.

Given the high prevalence of antimicrobial resistance in pigs in the DF, it would be desirable to implement a national antimicrobial resistance surveillance program similar to the Spanish model. On the other hand, considering the presence of antimicrobial resistance in commensals in pig samples in Brazil and Spain, it is desirable that the inhabitants of the commensal flora are also included in the ongoing microbial susceptibility monitoring.

Keywords: microbiota, epidemiologic surveillance, antimicrobial resistance, pigs.

Resumo

Título: Considerações sobre uso de antimicrobianos via oral em porcas e perfil de resistência fenotípica no microbioma nasal de suínos

Este estudo foi dividido em duas partes, ambas com o objetivo de avaliar o fenótipo dos habitantes da microbiota nasal suína. O primeiro estudo, realizado na Universidade de Brasília, consistiu no isolamento de agentes bacterianos coletados de swabs nasais de 50 matrizes suínas alojadas em 10 granjas comerciais com diferentes manejos sanitários no Distrito Federal, Brasil. Um total de 132 cepas cultivadas foram caracterizadas. A suscetibilidade microbiana foi avaliada pelo método qualitativo de Kirby-Bauer e estimou-se 55% de resistência antimicrobiana global (1573/2840 testes). Entre os 23 discos de antibióticos testados, bacitracina exibiu maior resistência (92%). *Pasteurella multocida* foi detectada em 60% das granjas, *Glaesserella parasuis* em 40% e *Actinobacillus pleuropneumoniae* em 10% das explorações. Não foi detectada a presença de M. *hyopneumoniae.* Foi encontrada uma associação positiva entre a redução das lesões no matadouro e a utilização de vacinas autógenas contra *P. multocida* (p=0,048). Agentes patogénicos como *Pseudomonas aeruginosa, Actinobacillus suis* e *Salmonella* sp. foram as espécies consideradas mais resistentes, mas não patogênicos, como *Staphylococcus* coagulase-negativo, também exibiram resistência.

O segundo estudo foi realizado no centro de pesquisa em saúde animal IRTA-CReSA. O método quantitativo de suscetibilidade antimicrobiana utilizado foi a microdiluição em caldo pela técnica de concentração inibitória mínima (CIM). Foi realizada a avaliação 56 estirpes obtidas a partir de isolados já disponíveis no laboratório. Os valores de CIM foram determinados para 24 antimicrobianos. Entre os antimicrobianos testados, a resistência fenotípica à clindamicina foi a mais frequente.

Tendo em conta a elevada prevalência de resistência antimicrobiana em agentes não patogênicos da microbiota nasal de suínos tanto no Distrito Federal, Brasil quanto em Barcelona, Espanha, sugere-se que agentes comensais também sejam testados e abordados no plano de resistência a antibióticos (PAN BR AGRO, Brasil; PRAN, Espanha). Por fim, a inclusão de estudos de caracterização de agentes bacterianos obtidos a partir de swab nasal de suínos, ampliando o escopo em curso, pode fortalecer o programa brasileiro para o monitoramento de suscetibilidade microbiana na abordagem Saúde Única.

Palavras-chave: microbiota, vigilância epidemiológica, resistência antimicrobiana, suínos.

Abbreviations

ABPA Associação Brasileira de Proteína Animal

AMB Antimicrobials

AMO Amoxicillin

AMR Antimicrobial resistance

AMU Antimicrobial use

AST Antimicrobial susceptibility test

BOPO6F Bovine/Porcine BOPO6F Vet AST Plate

CAPES Coordenação de Aperfeiçoamento de Pessoal de Nível Superior

CReSA Centre for Research in Animal Health

DF Federal District

ECOFF Epidemiological cut-off value

EUCAST European Committee on Antimicrobial Susceptibility Testing

GPN3F Gram Positive GPN3F MIC Plate

IRTA Institute for Agrifood Research and Technology

MDR Multidrug resistance

MIC Minimum Inhibitory Concentration

PCR Polymerase Chain Reaction

PRDC Porcine Respiratory Disease Complex

PROAP Postgraduate Support Program

R Resistant

S Sensitive or Susceptible to greater exposure (I)

SEAGRI Secretary of Agriculture of Federal District

UE European Union

UN Environment Programme (UNEP)

UnB University of Brasília

WHO World Health Organization

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INTRODUCTION

Recent surveys conducted by the Brazilian Animal Protein Association reveal robust growth in Brazilian pork exports (ABPA, 2024). To maintain its world-leading position, Brazil needs to adapt to international regulations and reduce antimicrobial use (AMU) on pig farms (Lekagul et al. 2019; EU 2019/6). To date, in Brazil, cannot be used as performance-enhancing additives: amphenicol, tetracycline, penicillin, cephalosporin, quinolone, sulfonamide, erythromycin, spiramycin, colistin, and more recently tylosin, lincomycin, and tiamulin (Lentz, 2022). Likewise, particular attention has been paid to antimicrobial (AMB) with critical importance for humans, such as third generation cephalosporins and fluoroquinolones (Collignon & McEwen 2019).

Indeed, inappropriate AMU in animals is a major driver of antimicrobial resistance (AMR) because misuse of AMB exerts selective pressure on the microbiome (Thompson et al. 2023), which favors the survival of resistant strains through the spread of resistance genes (Gostev et al. 2021; Zeon & Kibe 2023). Furthermore, resistance genes can be exchanged between animals, humans, and the environment (Collignon & McEwen 2019).

Moreover, commensal bacteria are the source of the AMR genes that are transmitted to human pathogenic bacteria through horizontal gene transfer (Salam et al. 2023). Besides, AMB mishandle has the potential to disrupt the beneficial microbial communities of the microbiota (Baele et al. 2001), leading to dysbiosis, an unfavorable imbalance in the composition and diversity of the microbiota (Elgamal et al. 2021). In addition, commensal bacteria play an important role in preventing the colonization of pathogens through competitive exclusion and the excretion of bacteriocins capable of bacterial lysis (Collins & Bowring, 2023). Dysbiosis of the normal microbial community increases the risk of pathogens (Caballero-Flores et al. 2023) involved in the porcine respiratory disease complex (PRDC) as *Actinobacillus pleuropneumoniae (APP), Pasteurella multocida, Mycoplasma hyopneumoniae (Myho), Bordetella bronchiseptica, and Glaesserella parasuis* (Brombilla et al. 2019). Thus, since the nasal microbiota regulates local immunity and contributes to the respiratory health of pigs, the issue should be addressed to reduce the microbiota detrimental effect and AMR (Alvarado et al. 2022).

Considering the new legislation on AMB, monitoring resistance patterns of the most common PRDC (Vilaró et al. 2020) is an important step in the context of One Health (EU 2023/27, Ferdinand et al. 2023; Murray et al. 2023). Thus, resistance monitoring has been emphasized to detect and follow the emergence of resistance in addition to providing veterinarians with data to optimize therapy (El Garch et al. 2016). Further, it contributes to the development of AMB stewardship and help to guide the therapeutic treatment (Holmer et al. 2019; Vilaró et al. 2020).

However, the AMR carried by pathogens involved in PRDC varies considerably in countries, regions and herds over time (Haimi-Hakala et al. 2017). Likewise, commensals may harbor AMR genes carried by healthy pigs (Argudín et al. 2015). Curiously, bacterial agents collected from non-diseased pigs in England had greater AMR prevalence for periods between 2009–2011 and 2013–2014 than cases of disease isolates (Hernandez-Garcia et al. 2017). Similarly, *S. suis* isolates from healthy and diseased pigs from Korea were resistant to at least one of the AMB tested (Gurung et al. 2015). Also, Zhang et al. (2015) indicated genetic complexity between herds and a close linkage among *S. suis* isolates from healthy sows and diseased pigs in China. Even before, Zhang et al. (2008) had described AMB susceptibility of *S. suis* isolated from clinical healthy sows.

Meanwhile, high resistance profiles of bacteria from pig respiratory microbiota have been isolated in Brazil (Serpa et al. 2020) as well as in Spain, the highest pig producer in Europe (Cameron-Veas et al. 2016; Vilaró et al. 2023; Uruén et al. 2024; Petrocchi Rilo et al. 2024). In addition, antimicrobial susceptibility testing (AST) of swine respiratory pathogens has been monitored in Europe (El Garch et al. 2016, Siteavu et al. 2023, Somogyi et al. 2023) and worldwide (Sweeney et al. 2011; Ke et al. 2024). This global scenario highlights the emergence of multidrug resistant (MDR) bacterial strains in livestock. The lack of susceptibility to at least one agent in three or more chemical classes of AMBs is referred to as MDR (Magiorakos et al. 2012). That is of concern not only from an animal health perspective, but also in terms of food safety and public health protection (Gostev et al. 2021).

Comparing European and Brazilian models, Brazil has made progress in the educational program for more conscious of AMU in livestock (Brazil, 2020), and even a resistance monitoring program is underway, but in its early stages (PAN BR AGRO, 2023). On the other hand, Europe has been monitoring the respiratory pathogens isolated from cattle and pigs through the VetPath program. VetPath is the first international AST program for food-producing animals in Europe using standardized methods and centralized broth microdilution determination (El Garch et al. 2016). MIC test is a better alternative to disc diffusion for surveillance programs (Somogyi et al. 2023; Gutiérrez-Martín et al. 2024), but disk diffusion assay (Hudzicki, 2016) is often used in Brazilian studies (Serpa et al. 2020) as well as in many other countries (Abdel-Moein et al. 2022; Bovo et al. 2023; Siteavu et al. 2023).

Therefore, it is essential to have epidemiological knowledge of the nasal colonizers in swine herds. It is also prominent to include commensals, as AMR patterns can also be present in non-pathogenic strains (Holmer et al. 2019). The origin preferably applied to sows (Vilaró et al. 2020), given that sows stay longer in the barn, they are more likely to have been exposed to most AMB

(Bosman et al. 222) as well as pathogens, making the sow stage significant to investigate the impacts of AMR (Alvarado et al. 2022).

OBJECTIVES

General Objective:

To identify bacterial agents in the nasal cavity of sows and describe the phenotypic resistance profiles of bacterial isolates to AMBs commonly used in swine production.

Specific Objectives:

1. To contribute to the epidemiological knowledge of agents collected from sow nasal swab in Federal District, Brazil.
2. To evaluate the resistance profiles of bacterial isolates through qualitative susceptibility testing.
3. To evaluate the resistance phenotypes in bacterial isolates available from IRTA-CReSA, Barcelona – Spain, using the minimum inhibitory concentration (MIC) microdilution technique.
4. Publication scientific literature providing guidance on alternatives to the use of AMB.
5. Encourage PAN BR AGRO to promote continuous monitoring of AMR in pig herds, including the use of nasal swabs in sow farms.

CHAPTER I- LITERATURE REVIEW

This chapter includes the review article published on Translation Animal Science:

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Animal Health and Well Being

Concerns about the use of antimicrobials in swine herds and alternative trends

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ABSTRACT

Pig productivity in Brazil has advanced a lot in recent decades. Specialized breeds are more vulnerable to pathogens, which has boosted the use of antimicrobials by farmers. The selective pressure generated favors the emergence of resistant bacteria, which compromises the effectiveness of this treatment and limits therapeutic options. In addition to increasing costs and mortality rates in the production system, public awareness of this issue has increased. The authorities have imposed restrictive measures to control the use of antimicrobials and have banned their use as growth promoters. This literature review highlights biosecurity and animal welfare to prevent pig diseases. Hence, we describe alternatives to the use of antimicrobials in pig production for the selection of effective non-antibiotic feed additives that help maintain good health and help the pig resist disease when infection occurs.

LAY SUMMARY

Antimicrobial resistance has been a threat worldwide. To achieve conscious use of antimicrobials in pig production, it is essential to understand best management practices, including the use of biotics. Currently, there is no suitable standard for improving pig's health status. Meanwhile, antimicrobials as growth promoters are banned in many countries, and antimicrobials important to humans should not be used in animals. This research describes the concerns about antimicrobials in pig herds and the alternatives for farm management that may help reduce pathogen challenge and mortality rates, maintaining animal performance indices. We bring results from scientific articles to describe methods that can be beneficial for the pork industry.

Key words: biosecurity, biotics, microbial resistance, swine, welfare animal

INTRODUCTION AND MOTIVATION

Since the discovery of their success as growth promoters in the 1950s, antimicrobials have been added to animal feed (Letek, 2020). Low doses of antimicrobials have also become commonly used to promote animal health and welfare, mostly in pigs and broilers (Valentim et al., 2019; Zeon and Kibe, 2023). Tetracyclines, macrolides, avoparcin, and penicillin are still commonly used in livestock farming to promote growth by increasing feed absorption, resulting in animal weight gain and improved herd health (Costa-Hurtado et al., 2020).

Since it was possible to achieve greater progress through genetic selection combined with better diet formulation (Patience and Ramirez, 2022), health challenges increased, and it has boosted the use of antimicrobials as growth promoters (Dutra et al., 2021). The widespread use of them has increased the microbiome selection pressure and engenders the spread of resistance genes among animals, humans, and the environment (Collignon and McEwen, 2019).

Although this practice has prevented illnesses' clinical signs, it can cause dysbiosis, characterized by an unstable microbiome, and might increase susceptibility to diseases caused by opportunistic microorganisms (Correa-Fiz et al., 2019). In this way, the unstable flora interferes with the animal's immunity, making it more susceptible to opportunistic agents that may carry resistance genes and no longer respond to the used antimicrobials (Costa-Hurtado et al., 2020). Therefore, unbalanced microenvironment is a bigger challenge, especially for piglets (Blanco-Fuertes et al., 2023).

The One Health approach published concerns about the microbiome selective pressure induced by antimicrobials (Collignon and McEwen, 2019). Some bacterial strains become able to escape the action of antimicrobials by drug efflux pump, enzyme production, and acquisition of resistance genes (Abavisani et al., 2021; Seo et al., 2023). The transfer of resistance factors between bacteria makes the challenge even greater.

In fact, to regain control of antimicrobial resistance (**AMR**), the governments from many countries approved restriction measures. In the absence of restriction measures, multidrug resistant bacteria (**MDR**) can then spread between and within animals, humans, and the environment. According to a standard of definitions proposed by Magiorakos et al. (2012), bacteria are considered MDR when they become resistant to at least one of three different classes of antibiotics used to treat infections. Public policy authorities around the world are encouraging a new culture among pig producers to

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achieve the conscious use of antimicrobials (Collignon and McEwen, 2019).

Antibiotic-free feed additives, the new generation of growth promoters, can consist of organic acids (Tugnoli et al., 2020), essential oils (Xiong et al., 2020), prebiotics (Wang et al., 2022), and probiotics (Yang et al., 2020). However, first and foremost, biosecurity (Alarcón et al., 2021) and welfare (Neila-Ibáñez et al., 2023) measures must be well established. Although biotics play a significant role in promoting improved immune systems and antioxidant status in animals, their effects vary depending on the facilities, genetics, health management, dose, frequency, and even the age of the pigs (Patience and Ramirez, 2022). Not all of them provide the same beneficial response in different herds (Angelakis, 2017), so it is relevant to consider the particular conditions of each pig farm when choosing an antibiotic-free feed additive.

Knowledge about the benefits of biotics related to improving the herd's immune system or increasing herd performance is vast; however, selecting effective measures to protect pigs against pathogens is a challenge. Would it be feasible not to use anymore antimicrobials in pig's feed and meanwhile keep them away from illness? Patience and Ramirez (2022) showed the importance of a holistic approach for the adoption of antibiotic-free pork production. Indeed, there are many ways to achieve this goal. Here, we describe the alternative trends against opportunistic pathogens in pig farms to reduce the spread of AMR.

ANTIMICROBIALS AS GROWTH PROMOTERS

In 1928, bacteriologist Alexander Fleming discovered that penicillin, a substance produced by a fungus, could inhibit the growth of bacteria. Together with scientists Ernst Chain and Howard Florey, the bacteriologist developed methods for the mass production of the substance and, in 1941, the first antibiotic was available to the human population and animals (Letek, 2020). It was then noted that the use of low doses of antimicrobials improved animal growth (Zhu et al., 2023).

However, antimicrobial use did not always reflect the sanitary condition or the real therapeutic needs, easily leading to overuse (Dutra et al., 2021) Products for well-being of domestic animals account for 60% of the veterinary pharmaceutical industry's turnover (Caselani, 2014). In addition to treating infections, drugs have come to be used to maintain the quality of animals' gastrointestinal flora and are continuously administered in feed in smaller quantities than those used for therapy or disease prophylaxis (Lin et al., 2015).

Antimicrobials control the pathogenic flora in the digestive system and thus reduce the competition for nutrients, as well as reduce the production of growth-depressing metabolites in animals (Brockmeier et al., 2012). In addition, antibiotic medications reduce the size and weight of the digestive tract, which makes the villi and intestinal walls thinner, increasing nutrient absorption and, at low doses, improving herd performance and zootechnical indices. However, continuous use gradually decreased the effectiveness of the drugs, which led to the need for higher doses (Angelakis, 2017).

The discovery of antimicrobial substances has effectively helped to prevent and treat infections; however, in the long term, it has led to the emergence of MDR (Laird et al., 2021; Wang et al., 2022; Liu et al., 2023). The increase of antimicrobial pressure due to its overuse (Hopman et al., 2019) has led o AMR and threats to global public health (Ferdinand et al., 2023).

ANTIMICROBIAL RESISTANCE: THE NATURAL PHENOME GAINS SPEED

According to Collignon and McEwen (2019), AMR is an ecological problem characterized by complex interactions involving various microbial populations that affect the health of humans, animals, and the environment. Hughes (2014) clarified that antimicrobial agents do not directly generate resistance, but exert selective pressure on a given bacterial population, favoring the emergence and growth of resistant bacteria.

Zeon and Kibe (2023) argued that new bacterial genotypes are rarely established and spread through random genetic selection resulting from the absorption of free DNA in bacteria classified as "competent," those that can absorb free DNA from dead bacteria, given that this characteristic is found in only around 1% of validly described bacterial species. Therefore, the occurrence of new bacterial genotypes by natural transformation is less frequent because the stability of DNA released into the environment and the ability to integrate into a replicating genetic element with a lack of DNA sequence similarity is limited (Thomas and Nielsen, 2005).

However, the chances of natural transformation increase in strains that have already mutated, since the dependence on DNA sequence similarity for recombination between species is relaxed in some mutant isolates (Abavisani et al., 2021). Furthermore, DNA acquisition through double-strand breaks and end joining—illegitimate recombination—applies more to the integration of circular DNA into linear fragments (Neves et al., 2007).

Mutations are more frequent due to horizontal gene transfer by transduction—transfer of bacterial DNA between a bacteriophage-infected bacterium and a bacteriophage-susceptible bacterium and by conjugation—and by transfer of mobile genetic elements by pili structures between two bacteria located adjacently. Thomas and Nielsen (2005) explained that for the transduction and conjugation mechanisms to flow, there is a dependence on the activity of the receptor restriction enzyme.

The interaction between microorganisms can increase the severity of infection of an opportunistic pathogen and co-infections by different strains can cause recombination and genomic changes (Ouyang et al., 2019). When administering an antimicrobial for the treatment of a respiratory condition, not only the colonies of bacteria causing the disease are affected; others not involved in the present infection can become resistant (Zeon and Kibe, 2023).

Notably, the circulation of resistant strains can occur in sick or healthy animals. Zhang et al. (2008) reported resistance of *Streptococcus suis* to antimicrobials in clinically sound sows in China. Gwida et al. (2020) identified *Escherichia coli* carrying the resistance gene shigella (stx1) in a healthy buffalo with the same genetic pattern in *E. coli* isolates from humans and animals, indicating the potential to become a source of genetic material exchange.

Dong et al. (2022) presented the most comprehensive transmission of bacteria with tigecycline inactivating enzyme, mediated by Tet(X) plasmid in animals, humans, and environmental niches in China. Tigecycline is the first drug of the glycylcycline class of antimicrobials, an antibiotic derived from tetracycline for use in humans. Tet(X)-positive bacteria

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were highly diverse and mult resistant. Isolates from different families exhibited different AMR profiles. In Dong's study, the coexistence of tet(X) with other resistance genes such as florfenicol was commonly observed (66.8%).

Enzyme production and enzyme modification is an important mechanism of resistance (Lin et al., 2015). Betalactamases (blaTEM, blaSHV, and blaCTX-M) and the acquisition of quinolone resistance genes (qnrA, qnrB, qnrS, and aac(6′)-ib) confer resistance to beta-lactam drugs and quinolones (Seo et al., 2023).

Another important mechanism in bacterial drug resistance is drug efflux. The MexXY efflux system, present in a variety of different bacteria, has contributed significantly to the increase in resistance to multiple antimicrobials (Abavisani et al., 2021). Efflux activity is mediated by a class of membrane protein transporters called multidrug efflux pumps, which actively expel a variety of cytotoxic substances, including antimicrobials, out of bacterial cells (Allen et al., 2010).

Usui et al. (2013) and Deng et al. (2013) have already confirmed that even a single bacterium can possess multiple efflux transporters from different families, with overlapping substrate spectra. Palmieri et al. (2011) and Huang et al. (2018) described a gene delivery system driving the evolution of AMR by *S. suis*.

Due to the sharing of resistance genes, numerous active ingredients have reduced their efficacy. Felde et al. (2020) reported single nucleotide polymorphisms in the parC (C239A/T and G250A) and gyrA (G242C, C247 T, and A260 G) genes that showed a correlation with decreased susceptibility to fluoroquinolones by altering the target site and added that the alteration of nucleotide A2059 G in the 23S rRNA sequence correlates with significantly decreased susceptibility to macrolides and lincosamides.

The flow of MDR can occur from animals to humans or the other way around but is not restricted to susceptible hosts, as they can be transmitted through food. In Brazil, *Salmonella* spp. is the second largest disease-causing agent involved in outbreaks of food-borne diseases (Santos et al., 2020).

In Europe, Barcala Salido et al. (2022) reported a rare clinical case of a pig farmer with pneumonia caused by *Bordetella bronchiseptica*, suggesting that even though there are limits to the host range of the mechanism of transfer and maintenance of mobile genetic elements (Thomas and Nielsen, 2005), prokaryotic organisms may not respect the boundaries between species.

Because of the resistance mechanisms, antibiotics have been less effective. Resistance to florfenicol in an isolate of virulent and the MDR *Pasteurella multocida* serogroup A from duck liver that contained a plasmid (pXL001) carrying the florfenicol resistance gene was reported by Yajuan Li et al. (2023).

In this context, cases of resistance to ceftiofur, a third-generation cephalosporin used to treat pneumonia in animals, have already been reported in *E. coli* and *Staphylococcus* sp. strains in various regions of Canada and Brazil (Costa, 2021). Peres et al. (2020) found that 10% of *Glaesserella parasuis* isolates showed resistance against gamithromycin (Zactran, Boehringer) with a minimum inhibitory concentration of ≥8 and ≤16 µg/mL. The macrolide antibiotic was recently placed on the market and had previously been successfully tested by Gupta et al. (2020) in an experimental *B. bronchiseptica* infection. Hamel et al. (2021) also observed recovery in pigs suffering from respiratory diseases treated with gamithromycin. Due to the resistance observed, Peres et al. (2020) suggested tildipirosin to treat respiratory disease in pigs.

Whenever he efficacy of a particular drug decreases, new antimicrobials are chosen and the pharmaceutical industry invests in the development of new active ingredients, but the process that will lead to resistance mechanisms is not interrupted (Caselani, 2014).

RESTRICTIVE CONTROL MEASURES: DEMAND FOR FREE ANTIMICROBIAL ALTERNATIVES

The United States and the European Union prohibit the use of antimicrobials in the production of animals that will be consumed by humans and implement strict controls on imported meat (FAO, 2022). Based on the recommendations of international reference organizations, Brazil implemented the National Plan for the Control of Residues and Contaminants—PNCRC and has restricted several antimicrobials as performance-enhancing additives, over the last few years. These restrictions began banning the use of avoparcin, arsenic, ammonia, chloramphenicol, nitrofurans, and carbadox.

To date, in Brazil, amphenicol, tetracycline, penicillin, cephalosporin, quinolone, sulfonamide, erythromycin, spiramycin, colistin, and more recently tylosin, lincomycin, and tiamulin cannot be used as performance-enhancing additives (Lentz, 2022). Education programs are a path for society to develop an awareness of AMR and to select alternatives to the use of antimicrobials. The World Organization for Animal Health has partnered with the Food and Agriculture Organization of the United Nations, the World Health Organization, and more recently, with the United Nations Environment Program to a worldwide campaign to raise awareness of the rational use of antimicrobials that encourages the use of alternatives instead (Huang et al., 2018).

BIOSECURITY: FIRST APPLIED MEASURES

The first applied measures are efforts to manage biological risk. It is designed to prevent the introduction and spread of disease within populations, herds, or groups of animals. Alarcón et al. (2021) classified the practices to minimize the risk of introducing exotic agents into the system as external biosecurity and those to reduce disseminating etiological agents within the herd as internal biosecurity. Identifying critical points involving external and internal biosecurity helps to design the mitigation measure, which is a risk-based surveillance guideline.

Well-designed vaccination program is considered an effective strategy for controlling respiratory diseases in pigs (Patience and Ramirez, 2022). However, even after diagnostic screening and protocols consistent with the circulation of local strains, immunization does not prevent pathogen colonization (Endale et al., 2022). In this way, biosecurity plays an essential role.

Since the highest probability of introduction of a new pathogen is the introduction of animals, one of the mitigation measures is quarantine (Maes et al., 2016). The Brazilian legislation preconize 500 m distance from other production sheds. Vehicles for animals separated from feed and disinfection arrows avoid cross contamination. Other measures to prevent pathogen introduction are fumigating all the stuff before getting on the farm, blocking the entry of those who have met other pigs in the last 72 to 24 h, and making sure everyone shies and changes clothes (Bhilegaonkar et al., 2024).

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The farm, isolated from wi d boards, and at least 2 miles from swine herds, and abattoirs, when rounded by green belt and maintained fences, is protected from sources that could carry pathogens. Indeed, rodent and insect control programs and microbiological water analysis are required. Monitor the cleaning distribution system, the chlorine in the water and effluent system eliminates most of the microorganisms in the environment. However, to reduce the pathogen microorganisms pressure, it is necessary to free the batches for at least 3 days for cleaning and disinfection. This challenge can be reduced with adequate animal density, facilitating routine cleaning and disinfection when adopting the "all-in/all-out" system (Rojo-Gimeno et al., 2018).

Care for newborn piglets increases their health status. Takeuti et al. (2023) recommended segregated farrowing for gilts to reduce the spread of the pathogen to litter. Gilts play an important role in the transmission of Mycoplasma hyopneumoniae to their offspring (Kuhnert and Jores, 2021) since a high proportion of gilts seem to eliminate the bacteria more intensely at first calving, compared to older nonprimiparous sows.(Replace Fano by Takeuti et al., 2023)

Lentz (2022) emphasized that the periodic application of a checklist auditing the biosecurity actions adopted in the production system is a practical way of measuring internal and external risks, allowing for the immediate correction and/or adoption of specific preventive actions. Indeed, staff need to know why and how they perform each task through established operating procedures and constant staff training.

Some practices focus on adopting a new, more conscious culture in all actors of the pig production chain, and not all of them necessarily require large investments or technology, but mainly changes in management practices after evaluating the cost–benefit, implemented when it is necessary (Collignon and McEwen, 2019).

Dutra et al. (2021) estimated that the average amount of antimicrobials used in Brazilian herds is 358.4 mg/kg of pigs produced and that seven drugs are used per pig herd. However, this data did not correlate with the biosecurity score or productivity. However, after the implementation of biosecurity measures in sanitary management, the consumption of antimicrobials was reduced. In their study, there was a 44.3% reduction in the lifetime exposure of pigs to antimicrobials and the average number of drugs used fell from seven to five.

Reviewing and considering management practices, adapting behavior, choosing facilities, and checking for situations that cause stress and disease are fundamental aspects to promote more balanced production (Patience and Ramirez, 2022). The biosecuri y program involves e onomic, sociological, and even psychological aspects (Bhilegaonkar et al., 2024). Unfortunately, many of these manuals have little real impact as producers think that those recommendations are irrelevant or impractical, even for those who have had disease outbreaks or may receive financial support. Part of this failure is due to the low confidence in government institutions. Thus, educational approaches should be used to reduce the prevalence of respiratory diseases on farms including strict compliance with biosecurity (Maes et al., 2016) and promoting animal welfare.

The applied measures of biosecurity to reduce the use of antimicrobials are listed in Table 1.

WELFARE ANIMAL: IMPROVED ANIMAL IMMUNE SYSTEM

Through best animal welfare practices the animals have their behavioral, environmental, and physiological needs met, and the herd is less stressed and less vulnerable to illness (Rojo-Gimeno et al., 2018; Pierozan et al., 2021). Neila-Ibáñez et al. (2023) demonstrated maintaining the ideal mean temperature in early nursery and the ideal mean of relative humidity in the farrowing unit to prevent *S. suis* outbreaks. Obregon-Gutierrez et al. (2021) recommended sow contact in piglet early life to shape the piglet nasal microbiota composition like healthy piglets. Lastly, Costa-Hurtado et al. (2020) stated that minimizing stressful situations, particularly in the weaning phase, prevents the development of Glässer's disease (Cerdà-Cuéllar et al., 2010).

Management and housing conditions reduce the infection disease risk factors (Neila-Ibáñez et al., 2023). To better distribute the feed, the dimensions for specific environments should be respected: density of 0.30 to 0.33 animals/m^2 in the nursery phase. To minimize lameness, the termination area should be 0.90 m^2/animal for the slatted floor and 1.0 m^2/animal up to 100 kg of live weight for a compact floor. For above 100 kg of live weight, 1.2 m^2/animal is required to reduce disputes over hierarchy (Brazil, 2020).

Research on animal welfare influences practice by identifying indicators of animal welfare influence management procedures. Pierozan et al. (2021) compared animal welfare indicators to feed conversion (**FC**) and daily feed intake in growing and finishing pigs (*Sus scrofa*). Better performance was obtained in the presence of positive social behavior, characterized as greater freedom to express natural behavior, such as sniffing/sniffing/licking, reflected in a lower prevalence of coughing ($P < 0.01$) and lameness ($P < 0.001$).

Table 1. Biosecurity measures to reduce the use of antimicrobials

Biosecurity measures	Effect
Vehicles for animals separated from feed and disinfection arrow	Avoid cross contamination
Quarantine, fumigation chamber, guest book, shower, and clothes changed	Prevent pathogen introduction
Control of pests, rodents, and insects	Reduces the entrance of pathogens
Water quality source, cleaning distribution system and effluent system efficient	Reduce the microorganisms pressure
Free the batches for at least 3 d for cleaning and disinfection	Eliminate most of the microorganism in the environment
Care for newborn piglets	Increase the health status
Farm manual	Standard operating procedures
Checklists	Record the monitoring

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Meanwhile, greater express on of negative social behavior (aggressive interactions) showed a positive statistical association with the presence of hernias ($P < 0.01$), lower consumption of feed and water ($P < 0.001$), and a higher prevalence of animals with body wounds ($P < 0.05$).

It is worth stating that promoting animal welfare goes beyond animal productivity. Pig production in intensive environments must meet the demands of the modern consumer, who not only wants the sensory attributes of the meat but also compliance with ethical issues related to animal welfare, the quality of life of the people involved, and care for the environment. Increasingly, networked and well-informed consumers are putting pressure on production chains to assume high standards of sustainability (Patience and Ramirez, 2022).

Consumers are becoming increasingly discerning when it comes to choosing products and, to this end, they demand information about the origin of the food, the use of preservatives, and the health and welfare standards offered to farm animals. In this scenario, how animals are raised, housed, transported, and slaughtered plays a central role in the process. For this reason, animal welfare has gone from being a mere added value to the product to becoming a mandatory criterion for those who want to remain competitive in the market.

Reviewing management practices, new conducts, training, monitoring activities, and identifying unsatisfactory situations is urgent and it helps to advance balanced production and healthy status. Providing a basic understanding of the needs and nature of animals requires consciousness to bring up a new culture in the operating system and educational approaches (Obregon-Gutierrez et al., 2010). Welfare Animal practices are listed in Table 2.

BIOTICS: THE NEW GROWTH PROMOTER GENERATION

The health disease process is multifactorial. Pig producers and veterinarians need solutions to reduce dependence on antimicrobials and minimize the health threat posed by the spread of AMR through the food chain (Cao et al., 2015; Laird et al., 2021). The solution may be closer than the producer realizes, with an integrated adaptation of the management system. Good production practices, animal welfare, biosecurity programs, and new nutritional management formulas can strengthen the immune system, even without injectable immunogens.

The success can only be truly achieved when all aspects of diet formulation and animal management are adequately addressed (Patience and Ramirez, 2022). Recent research suggests that previously unused additives are now essential because they play a similar role to growth promoters (Jarosova et al., 2023; Liu et al., 2023; Sanches et al., 2023). In this way, to promote beneficial microbial colonization in animals (Zhu et al., 2023), biotic is being used as alternatives to antimicrobials to increase gain weight (Angelakis, 2017) and to trigger host immunity against a specific pathogen (Blanco-Fuertes et al., 2023).

Therefore, organic acids (Matsui et al., 2021), plant extracts (Xiong et al., 2020), prebiotics (García et al., 2021), and probiotics (Yang et al., 2020) are based on the principle of symbiosis within reciprocal benefits (Angelakis, 2017). Whatever the biotic used, it must ensure an adequate balance of the microbiota, reflecting positively on the general state of health of the animal, with consequent influences on performance, in the carcass and meat quality (Sun et al., 2020).

ORGANIC ACIDS: THE GOOD GUYS!

Organic acids such as formic, propionic, acetic, lactic, sorbic, phosphoric, citric, benzoic, and fumaric acids have different acidification capacities and, depending on their concentration, different antimicrobial activities with the ability to modulate the microbiota of the breeding stock (Jarosova et al., 2023; Liu et al., 2023).

The use of blends can minimize the occurrence of genitourinary infections in sows and improve the performance of growing pigs (Matsui et al., 2021), as well as controlling or preventing infections by enterobacteria (*Sal. enterica*, *E. coli*, *Clostridium perfringens*, and *C. difficile*).

Ren et al. (2019) evaluated the effects of formic and propionic acids added to pig feed (1% mixture of 64% formic acid and 25% propionic acid) and observed that their use in the diet increased fecal consistency and minimized the occurrence of enteric problems. The fecal pH values, total fecal coliform count, and fecal *Lactobacillus* count were not significantly different between the groups.

The addition of various organic acids to feed has been studied for its effects on reducing the symptoms caused by ETEC infection in weaned piglets (Diao et al., 2019). Acidic conditions also increase the physiological functions of the gastrointestinal tract by activating the secretion of enzymes, including pepsin, chelating minerals (Tugnoli et al., 2020), subsequently increasing feed digestion (Ren et al., 2019) and daily weight gain, FC and protecting against infection diseases by maintaining intestinal barrier function (Silva et al., 2023). The organic acid blends are presented in Table 3

Table 2. Welfare animal practices

Welfare animal practices	Effect
Maintain ideal mean temperature in early nursery and ideal mean of relative humidity in the farrowing unit	Prevent *S. suis* outbreaks
Sow contact in piglet's early life	Shaped the piglet nasal microbiota composition like healthy piglets
Minimize stressful situations, particularly on the weaning phase	Prevent the development of Glässer's disease
Density of 0.30 to 0.33 animals/m² in the nursery phase	Distributes the feed equally
Slatted floor in termination: 0.90 m²/animal; if compact floor: 1.0 m²/animal up to 100 kg of live weight	Reduce dispute over hierarchy
Freedom to express natural behavior such as sniffing/sniffing/licking	Lower prevalence of coughing and lameness

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ESSENTIAL OILS: SECRETS OF THE PLANT EXTRACTS!

Essential oils are extracted from plants with antimicrobial, antioxidant, and anti-inflammatory effects which regulate palatability and modulate a beneficial microbiota in swine herd (Peng et al., 2019). Thymol, carvacrol, and eugenol enhance sow´s palatability, increase feed consumption, regulate gastrointestinal function and reduce diarrhea in piglets (Biggs et al., 2020; Le et al., 2020; Xiong et al., 2020).

Plant extracts reduce immune stress by increasing enzymes such as catalase, superoxide dismutase, and glutathione peroxidase (Xiong et al., 2020), minimizing heat stress increasing interleukin IL-1b (Le et al., 2020) reducing circulating glucose concentrations (Biggs et al., 2020). It favors the firmicutes population and decreases the harmful strains in the colon (Peng et al., 2019).

As presented in Table 4, the blends may have benefits on the immune system and/or enhance gastrointestinal function.

PREBIOTICS: IT WAS NOT SUPPOSED TO EAT, AT FIRST!

Prebiotics are nonliving products that are not digestible but fermented by the host microbiota to change the composition of the intestinal flora. They are functional oligosaccharides that develop a healthy microbiota improving the growth performance of animals (Angelakis, 2017). Those oligosaccharides different molecular structures wield bacteriostatic properties (Yu et al., 2022) selectively favor the growth of beneficial bacteria such as *Lactobacillus* (Yang et al., 2020) and *Bifidobacterium* (Feng et al., 2019).

Liu et al. (2023) presented bacteriostatic properties of oligosaccharides from a wide variety of sources, such as different fruit peels, including mango, apple, and citrus fruits. At present, the most widely used are fructooligosaccharides (Ortega-Gonzalez et al., 2014), chitosan (Yu et al., 2022), chitin (García et al., 2021) and mannose-binding lectin (Fadl et al., 2021). Meanwhile, dietary proto ol has been widening with pectin (Wang et al., 2020), xylitol (Liu et al., 2023), xanthan hydrolysates (Wang et al., 2020), glycosidic bonds ((Liu et al., 2023), milk (Spicer et al., 2022), alginate oligosaccharides (Kaour et al., 2024), and agaro-oligosaccharides (Jiang et al., 2021).

Oligosaccharides prebiotics possess antioxidant and antibacterial properties and may also labor for breeding and nursery animals to adsorb mycotoxins (Yu et al., 2022); and for finishing animals to control *Clostridium* spp. and minimize the occurrence of mesentery torsion (Kaour et al., 2024). Prebiotics as an alternative additive to reduce the use of antimicrobials are listed in Table 5.

PROBIOTICS: DO ONLY GRAM-POSITIVE STRAINS PROMOTE BENEFICIAL EFFECTS?

Probiotics are beneficial bacteria that help to strengthen the immune system (Wang et al., 2020). The use of probiotics in agriculture predated the use of antimicrobials. In the 1940s, the use of *S. aureofaciens* resulted in weight gain in animals. Probiotics used in animal feed are mainly bacterial strains of gram-positive bacteria and have been effectively used for weight gain in chickens, pigs, ruminants, and aquaculture (Angelakis, 2017).

Because probiotics vary in composition, Costa-Hurtado et al. (2020) believed that the balance between colonization and immunity is the key factor for success. Zhang et al. (2020) reminded the concentration of these microorganisms also varies, requiring a minimum dose of approximately three billion viable microorganisms to properly colonize the intestines.

The effect of probiotics on the digestive flora is probably caused by bacteriocin production (Chen et al., 2019). The regulatory effects on pro-inflammatory gene expression and cytokine production in piglet intestinal cells (Abouloifa et al., 2020) may antagonize pathogen growth and influence the

Table 3. Organic acids as alternative additives to reduce the use of antimicrobials

Organic acid additive	Effect
Propionic acids added to pig feed (1% mixture of 64% formic acid and 25% propionic acid)	Increased fecal consistency and minimized enteric problems
Formic/propionic acid at 1% weaning pigs' diet	Reduced the challenge with ETEC K88
Sodium butyrate in 0.2% growing-finishing pigs dietary	Beneficial to gut development
Acid calcium soap	Decreased the abundances of *E. coli* and *Campylobacter* spp.(feces)
Succinate acid (diet supplemented with 1% succinate)	Improve intestinal morphology of pigs fed a diet with 1% succinate
Sodium butyrate, medium-chain fatty acids, and *n*-3 polyunsaturated fatty acids in 7.75 g/kg sow dietary	Shortened the weaning-to-estrus interval and decreased the incidence of diarrhea in suckling piglets
Medium-chain fatty acid salts distilled from coconut oil	Decreased the intestinal colonization of *Salmonella*, *E. coli* in piglets

Table 4. Essential oils as alternative additives to reduce the use of antimicrobials

Plant extract additive	Effect
Thymol, carvacrol, and eugenol	Enhance palatability, increase feed consumption's sow, and reduce diarrhea in piglets
Icariin	Increased average daily gain in 1 g/kg in the newborn piglets dietary
Eucommia ulmoides leaf extract	Decreased the diarrhea rate by 0.5% in the weaning pigs dietary
Capsicum oleoresin	Reduced circulating glucose concentrations in 0.1 g/kg in the gilts and barrows dietary

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Table 5. Prebiotics as an alternative additive to reduce the use of antimicrobials

Oligosaccharide additive	Effect
Fructooligosaccharides	Reduce bacterial adhesion to the intestinal mucosa of piglets
Chitosan oligosaccharides	Antimicrobial properties
Chitin oligosaccharides	Improve defense against pathogenic infections
Mannan-oligosaccharides	Protection against *E. coli* in broiles
Pectic oligosaccharides	Protection against *Micrococcus luteus*
Xylo-oligosaccharides	Inhibit pathogenic and spoilage yeasts
Xantho-oligosaccharides	Protection against *Sta. aureus*
Glycosidic bonds	Protection against *Manheimia haemolitica*
Milk oligosaccharides	Protection against *Streptococcus* spp.
Alginate oligosaccharides	Inhibit the survival of harmful bacteria by regulating biofilm formations, and promoting phagocytosis
Agaro-oligosaccharides	Effectively reducing the relative abundance of harmful bacteria

Table 6. Probiotics as an alternative additive to reduce the use of antimicrobials

Probiotic additive	Effect
L. reuteri	Increase the colonization by *Lactobacillus*
L. salivarius	*Ruminococcaceae UCG-014*, *Bacteroides*
L. rhamnosus GG	Reduced the inflammatory responses in preterm gnotobiotic piglets
L. plantarum strain JDFM LP11	Increase the colonization by Firmicutes, Spirochaetes in gut, IgG (serum)
L. delbrueckii	Reduced the oxidative response in weaned piglets in intestine
Sac. boulardii	Increase the colonization by Turicibacter, Ruminococcaceae_UCG_009 in gut
Bif. animalis	Modulate gut microbiota, enhance antioxidant capacity
E. faecium	Increased weaning weight, average daily gain, and gain:feed ratio

susceptibility of pigs to pathogen colonization (Zhang et al., 2020).

To date, the genera *Lactobacillus* (Shin et al., 2019; Splichalova et al., 2019; Yang et al., 2020), *Sacchromyces boulardii* (Zhang et al., 2020), and *Bifidobacterium* (Spicer et al., 2022) favor the growth of beneficial bacteria in weaned piglets, meanwhile, *Enterococcus* genera are the most important for suckling (Lan and Kim, 2020) and postweaning piglets (Zhang, 2020), Finally, *Bacillus subtilis* is more effective in colonizing the animals' intestines in later stages, but is manly used in broilers (Ji et al., 2022; Šimunović et al., 2022).

However, it is questionable whether gram-negative colonizing healthy piglets' respiratory tract can play a role as a potential probiotic (Arriba et al., 2018; López-Serrano et al., 2020). Recent studies observed distinct colonizers which modulate the nasal microbiota in healthy piglets (Blanco-Fuertes et al., 2023). Arriba et al. (2018) characterized the genus *Bergeyella* spp., gram-negative strain, isolated from the nasal cavities of piglets. Although some showed in vitro features indicative of a virulence potential, the inoculated were passages from healthy piglets and two of those farms did not use antibiotic treatment.

Along these lines of research, López-Serrano et al. (2020) have shown genus *Moraxella* spp. also gram-negative strain, is commonly present in the nasal microbiota of swine and revealed heterogeneity among *Moraxella* spp. strains. Strains with pathogenic potential were detected as well as those that may be commensal members of the nasal microbiota. These studies suggest the role of *Bergeyella* spp. and *Moraxella* spp. in porcine diseases and health should be further evaluated.

Finally, Table 6 lists the microorganism probiotic and the protection effect on the host.

CONCLUSION

The search for solutions to the problem of AMR considers this complexity and ecological nature through a coordinated and holistic approach. We have shown that encouraging new alternatives helps to achieve sustainable development goals. One measure of success in reducing antimicrobials in pig herds can be educational approaches, as the proportion of antimicrobials used on Brazilian farms has been reduced following improvements in sanitary management.

Biosecurity, animal welfare, and biotics expand the possibilities for the pig industry, increasing efforts to deal with the threat posed by AMR. Perhaps there are more possibilities for bacterial genes that can favor the growth of beneficial bacteria in weaned piglets that will become available in the future. Although much remains to be clarified, encouraging the adoption of new alternatives is a step towards reducing the use of antimicrobials on pig farms.

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Conflict of interest statement

None declared.

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CHAPTER II- UNB RESEARCH

Title: Nasal phenotypic resistance profile of sows under different health managements in Federal District, Brazil.

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Abstract: Antimicrobial resistance is a universal threat and is leading to a new awareness of antimicrobial use. The colonization of tissues by some microorganisms carrying resistance genes may pose a risk of spreading resistance to pathogens. Antimicrobials may induce an unstable microbiome that compromises the animal's immunity. Indeed, dysbiosis has been linked to many alterations in the immune response. Here, we isolated bacterial colonizers from the nasal microbiota of sows to describe the phenotypic resistance profile on different health managements. One hundred and thirty-two strains isolated from 50 nasal swabs collected from sows were tested against up to 23 antibiotics by disk diffusion. Overall, the nasal communities showed 55% antimicrobial resistance (1573/2840 tests). Bacitracin showed the highest antimicrobial resistance (92%), followed by florfenicol (76.5%). Pathogens such as *Pseudomonas aeruginosa, Actinobacillus suis* and *Salmonella* ser. Typhimurium were the most resistant species, but the commensal nasal microbiota also presented antimicrobial resistance. Bearing in mind the high prevalence of antimicrobial resistance, the implementation of a national antimicrobial resistance surveillance program would be desirable for future evaluation of interventions through more conscious measures in the use of antimicrobial agents highlighting animal welfare, biosecurity, good production practices and alternatives to antimicrobial use in pig farms.

Keywords: antimicrobial resistance; dysbiosis, respiratory, microbiota, swine.

Introduction

Four decades ago, antimicrobials at low dosages used to be part of swine herd management to improve animal weight [1]. Nowadays, the use of growth promoters and metaphylactic treatment is still a practice in broiler chickens and pigs [2]. In Brazil, antimicrobials are also used for enhancing animal health by reducing the burden of pathogenic microbes and, therefore, disease outbreaks in swine herd [3].

Nonetheless, the widespread use of antimicrobials has increased the selection pressure on the microbiome and promoted the spread of resistance genes among animals, humans and the environment. The emergence of antimicrobial resistant microorganisms is a consequence of the continued use of antimicrobials, increasing costs and mortality rates in the production system, and causing a global health problem [3,4]. Antimicrobial resistance (AMR) occurs when a microorganism develops mechanisms that allow the growth in the presence of antibiotics. A microorganism that has acquired resistance to at least one agent in three or more antimicrobial categories is characterized as multidrug resistant (MDR) [5]. Thus, antimicrobial use puts pressure on a given bacterial population and selects for resistant variants [6], not only in the target pathogens but also in bacteria from the microbiota [7]. AMR compromises treatment efficacy and limits therapeutic options [8] and favors MDR colonization [5].

Therefore, antimicrobial treatment should only be recommended by veterinarians and in limited situations, mainly to treat piglets during a disease outbreak, which is important not only for health but also for welfare issues [6-8]. Reduction of antimicrobial use in veterinary medicine includes vaccination protocols and strategies to maintain a balanced microbiota through beneficial microbiota colonization [9]. Instead, pigs are fed high levels of antimicrobials [10]. Therefore, antimicrobial use (AMU) should be optimized to reduce the selection and spread of AMR [11]. It is important to improve managers' understanding of effective strategies to prevent AMU [12] and to maintain a stable microbiota community [13].

One of the major functions of symbiotic microorganisms is to protect against pathogens, mainly through pathogen exclusion and immune system stimulation [14, 15]. In this way, the nasal microbiota is an important contributor to respiratory health [13]. A dense and diverse microbial community inhabits the gut and many mucosal surfaces [16]. One of the strategies to ensure beneficial microbial colonization of animals [9] is to promote a balance between colonization and immunity [7].

Nevertheless, metaphylactic antibiotic treatments have the potential to disrupt the beneficial microbial communities of the microbiota, leading to dysbiosis [7, 17]. This unbalanced

microenvironment is more susceptible to pathogen invasion, raising the risk of disease development. Furthermore, if the microbiota members possess resistance genes, these can be transferred to the pathogens, potentially undermining disease management. [18]. Bacteria have the potential of genetic modification, and this will enable them, sooner or later, to neutralize the action of newly invented antibiotics [19].

Notably, the circulation of resistant strains can occur in sick or healthy animals. Antimicrobial resistance of *Streptococcus suis* in clinically healthy sows has been reported previously in China, and transmission is likely to occur between healthy carrier sows and their offspring [20].

Increasingly, networked and well-informed consumers are putting pressure on production chains to adopt high standards of sustainability [21]. Raising animals in good welfare conditions can strengthen the immune system and reduce the need for antimicrobial use. Good animal husbandry meets the behavioral, environmental and physiological needs of animals, resulting in less stress on the herd and less susceptibility to disease [22]. In this context, biosecurity should be improved by reviewing management practices [23] rather than relying on growth promoters and antibiotic treatments, which can lead to loss of drug efficacy [24].

To regain control of AMR, governments in many countries have approved restrictive measures. Public authorities are encouraging alternative practices among pig producers as the use of tetracyclines, macrolides, avoparcin and penicillin as growth promoters is prohibited. Since 2018, Brazil has established procedures for the production and use of veterinary drugs in feed, including the obligation to inform about the use of antimicrobials in feed formulation [25, 26]. To date, Brazil has not implemented a national antimicrobial resistance surveillance program. Antimicrobial resistance surveillance programs have become essential to control the use of antimicrobials in food production and to evaluate the strategies adopted. Government policies are promoting alternatives to AMU, enforcing animal welfare and good production practices, and a new culture that comes with microbial diversity [8].

Antimicrobial susceptibility testing (AST) is recommended for the appropriate use of antimicrobials [27] and to even identify MDR in commensal communities as an option to control the situation. The aim of this study was to isolate bacterial colonizers from the nasal microbiota of sows to describe the phenotypic resistance profile in different sanitary management areas in the Federal District, Brazil.

Materials and Methods

Ethical Approval

All samples were collected according to ISO/TS 34700:2016 with the permission of the farm owners. The study was approved by the Ethical Committee for the Use of Animals (CEUA) of the University of Brasília nº 23106.022976/2023-55. Certificate is in the Annex section.

Data collection from the swine farms and sows

From March 2022 to October 2023, sows were screened in 10 sow farms (A to J) located in the Federal District, Midwest Brazil, classified as two-site herds (1 farm), farrow-to-finish (3 farms), and one-site herd (6 farms). Herd size ranged from 50 to 4,273 sows. Five sows (14-48 months old) with cough and prostration were preferentially selected for sampling. In the absence of clinical signs, sows with non-clinical signs were selected to achieve 5 sows sampled. Therapeutically medicated sows up to seven days prior to the farm visits were not sampled. Therefore, a total of 50 nasal swabs in duplicate were placed in plain Falcon tubes for PCR assays and in 5 mL BHI, KASVI® tubes for bacterial culture.

Biosecurity Data Collection

The farms varied in animal welfare and sanitation management. The biosecurity data collection included herd, farm and owner information. Biosecurity was scored using 10 questions that assessed the risk of disease entering and spreading in the herd. The biosecurity score (Bio) ranged from 6 to 9 on a scale of 1 to 10 points and analyzed the preventive measures in place on the pig farms. The higher the score, the better the preventive measures in place. The main measures assessed were isolation of the farm, safe distance from other pig herds and roads, replacement of breeding stock, quarantine, possible sources of vectors, type of feed, mode of transport and registration of access by vehicles and people. The questionnaire used for this study was based on the official form of the Brazilian Ministry of Agriculture, and the reports are shown in Table 1.

Table 1 – Farm biosecurity evaluation by scoring preventive measures

Farm	F. isol.	Herds dist.	Road	Breed	Quaran	Vector	Feed	Trans	Vehicle disinf	Access	Bio
A	1	0	0.5	1	0.5	1	1	1	1	1	**8**
B	0.75	1	0.75	1	0	0.25	1	1	0.25	1	**7**
C	1	0.75	0.25	1	1	1	1	1	1	1	**9**
D	0.5	1	0.75	0.5	0	0.5	1	1	0.75	1	**7**
E	1	1	1	0	0	1	1	1	0	0	**6**
F	0.5	1	0.75	1	0	0.5	1	1	0.25	1	**7**
G	1	1	1	0	0	1	1	1	0.5	0.5	**7**
H	0.25	1	0.75	1	0	1	1	1	0.5	0.5	**7**
I	1	1	0.25	1	0	1	1	1	0.25	0.5	**7**
J	1	0	1	0	0	1	1	1	0.5	0.5	**6**

*F: Farm; F isol.: Farm isolation;n Herd dist.: Swine herds distance; Road: Road distanc;e Breed: Breeders reposition; Quaran: Quarantine; Vecto: Vectors control Feed: Type of Feed; Trans: Feed Transport; Vehicle disinf: Vehicle disinfection; Access: Human Access; Bio: Biossecurity score

The same researcher interviewed and collected data in all participating herds. The cross-sectional analytical study covers the population of 9,544 sows. They used different antimicrobials in the sow feed and the vaccination protocols included commercial and autogenous vaccines for several swine diseases. Qualitative and quantitative data on the health status of the herd in terms of management, structure and vaccines used were evaluated. The piglets were suckled for 21-24 days and then rigorously cleaned with disinfectant and lime. Although there are no mandatory vaccines, the farms used commercial and autogenous vaccines with different protocols.

DNA extraction and PCR assays

Genomic DNA was extracted from each strain using the Genomic DNA Extraction Kit® (Biogene, Madison, WI, USA) according to the manufacturer's instructions. PCR assays were performed as in previous work [28]. We detected *Actinobacillus pleuropneumoniae, Glaesserella parasuis*, and *Pasteurella multocida* by multiplex PCR assay and *Mycoplasma hyopneumoniae* by nested PCR [28]. The positive control samples were kindly provided by the Federal University of Viçosa - UFV and the negative control was ultrapure water.

Culture and Bacterial isolation

Standard microbiological techniques were used to isolate bacterial colonies at the Veterinary Microbiological Laboratory of the University of Brasilia (UnB). Nasal swabs were plated on blood agar and incubated overnight at 37°C. Morphology, hemolytic activity, Gram stain, catalase and oxidase tests were performed individually. Contaminating Gram+ bacilli with the presence of spores and yeasts were discarded. All colonies were classified according to the results of oxidative/fermentative (OF), methyl red, and Vöges-Proskauer (Vm/Vp) tests, followed by other biochemical tests according to the protocols established in the Standard Operating Procedure of the Veterinary Microbiology Laboratory of the UnB. In addition to Vm/Vp, staphylococci were cultured on salt-mannitol agar (BD®) and differentiated using the coagulase test. For enterobacteria, the characterization followed the established differentiation protocol: indol, citrate, urea and TSI (triple sugar iron) and consumption of sugars and proteins. After bacterial characterization [29], each strain was individually plated on blood agar and frozen using BHI with 20% glycerol.

Antimicrobial Susceptibility Test

Isolates were subjected to AST using the disk diffusion Kirby-Bauer method to evaluate phenotypic resistance [30]. The inhibition zone sizes around the antibiotic discs were read with a pachymeter to classify the results as resistant (R) or sensitive (S). Intermediate isolates that were susceptible to greater exposure were classified as sensitive (S). Interpretation of the resistance profiles was performed according to CLSI (Clinical and Laboratory Standards Institute) standards [31], where available, and the antibiotic manufacturer. The products have analytical sensitivities at their respective concentrations adjusted to McFarland's Standard Turbidity Scale 0.5. The antimicrobials used are listed in Table 2.

Table 2. Antimicrobials for susceptibility testing and their concentration

Pharmacologic Class	Antimicrobials	Concentration disk
Aminoglycoside	Amikacin (AMI)	30 µg
	Gentamicin (GEN)	10 µg
	Neomycin (NEO)	30 µg
Amphenicol	Florfenicol (FLF)	30 µg
β-lactamase	Amo + Clavulanic acid (AMC)	20 µg
	Amoxicillin (AMO)	30 µg
	Ampicillin (AMP)	10 µg
	Penicillin (PEN)	30 µg
Cephalosporine	Cephalothin (CFL)	30 µg
	Cephalexin (CFE)	30 µg
	Ceftiofur (CFT)	30 µg
Quinolones	Enrofloxacin (ENO)	5µg
	Marbofloxacin (MBO)	5µg
	Norfloxacin (NOR)	10 µg
Lincosamide	Clindamycin (CLI)	2 µg
Macrolide	Erythromycin (ERI)	15 µg
	Tylosin (TLS)	60 µg
	Tulathromycin (TUL)	30 µg
Tetracycline	Tetracycline (TET)	30 µg
	Doxycycline (DOX)	30 µg
Polypeptide	Bacitracin (BC)	10 µg
Sulphonamide	sulfametoxazol (SUL)	300µg
	sulfametoxazol-trimetoprim (SUT)	25 µg

Statistical Analyses

Graphs were generated with RStudio (2024.04.0) [32] using the ggplot2 package [33]. For each antimicrobial, the proportion of resistant isolates was calculated by dividing the number of resistant isolates by the total number of isolates tested. The association between AMR and genotypes and the origin of the isolates were statistically analyzed using Pearson´s chi-square test and the likelihood ratio, as performance before (Uruén et al. 2024). We chose a nonparametric test, Chi-Square, to estimate correlations between antimicrobial resistance and health management variables, such as antimicrobials used and vaccination protocols. Although Chi-Square is indicated to find a dispersion value for two nominal categorical variables, Fisher's exact test was used whenever the sample size was small to determine the exact probability of occurrence of an observed frequency. Associations between AMR, the genotype of isolates, geographical distribution, age of animals, and year of isolation were analyzed using Pearson’s chi-square test, however, when comparing two categories and more than 20% of the cells had a frequency lower than 5 units, the likelihood ratio test was

used. Associations were considered statistically significant when the p-value was lower than 0.05. In addition, adjusted standardized residues (ASR) were calculated and analyzed. When the ASR value is higher than 1.96, the frequency is significantly higher than expected and the relationship is considered positively significant; if the ASR value is lower than -1.96, the frequency is significantly lower than expected, and the association is considered negatively significant. When the ASR value is between -1.96 and 1.96 the association between variables is not statistically significant (Uruén et al. 2024). Statistical analyses were performed using SAS software (version v9.4, Cary, North Carolina, USA).

Results

Swine farms and sows' health management

Samples were collected from sows of different parity, with 42% (21/50) in first parity. Fifty-four percent (27/50) of the sows were in maternity stalls and 46% were in gestation stalls. Forty-four percent (22/50) of the sows showed no clinical signs of infectious disease, but a history of infectious disease was reported in 34% (17/50) of the sows. During the visits, 22% (11/50) of the sows were coughing, sneezing and/or had purulent or sanguinolent nasal secretions.

The number of workers varied from 1 to 96 depending on the herd size, but the number of sows per worker varied from 27 to 65.75 among the herds, with an average of 46.3 sows per worker. The smallest herd of 50 sows was managed by only one worker, while the largest herd of 4,273 sows was managed by 96 workers. Some farms used in-feed amoxicillin continuously, while others used antibiotics on a rotational basis in the sow feed.

All farms added amoxicillin to sow diets, but some farms added it with florfenicol, penicillin or tylosin. Farm managers used 800 mg/ton to achieve 20 mg/kg per lactating sow, while lower doses were used as growth promoters for gilt acclimation. Farm A frequently included clindamycin, tetracycline, enrofloxacin and oxytetracycline in the feed rotation for metaphylactic purposes, and farm J included clindamycin. Occasionally, marbofloxacin, gentamicin, or amoxicillin with clavulanic acid were injected for therapeutic treatment. Amikacin's use was not reported by any of the farm managers or staff.

The vaccination protocols varied among the farms. Commercial vaccines were used in 7/10 farms and were directed against *Mycoplasma hyopneumoniae* (Myo), porcine circovirus type II (PCV2), *Pasteurella multocida, Bordetella bronchiseptica, Salmonella* ser. Typhimurium, Streptococcus suis infection diseases. Autogenous vaccines were used in 8/10 herds to prevent

outbreaks caused by *P. multocida, Glaesserella parasuis, S*. Typhimurium, *E. coli* and, *S. suis*. Table 3 details health management practices among swine farms, including metaphylactic feed and vaccine protocols adopted by each farm.

Table 3. Farm, antimicrobial agents in metaphylactic treatment, vaccines protocols and production type.4).

Farm	Antibiotics	Vaccines	Production Type
A	AMO, CLI, TET, ENO, OXY	*M. hyopneumoniae, circovirus, P. multocida, S.* ser. Typhimurium	One-site-herd: piglet unit production
B	AMO, FLF, PEN	*M. hyopneumoniae, circovirus, G. parasuis, S. suis.*	One-site-herd: piglet unit production
C	AMO, FLF	*P. multocida, B. bronchiseptica, G. parasuis, S.* ser. Typhimurium, *E. coli.*	Two-site-herd: piglet and gilt and young boar production
D	AMO, FLF	*G. parasuis*	One-site-herd: piglet unit production
E	AMO	*None*	Farrow-to-finish: piglet to hog-finished production
F	AMO, FLF, PEN	*P. multocida, S.* ser. Typhimurium, *S.suis.*	One-site-herd: piglet unit production
G	AMO	*None*	Farrow-to-finish: piglet to hog-finished production
H	AMO, FLF	*M. hyopneumoniae, circovirus, P. multocida, S.* Typhimurium, *S. suis*	One-site-herd: piglet unit production
I	AMO, FLF, TYL	*M. hyopneumoniae, circovirus, P. multocida, G. parasuis, S.* ser. Typhimurium	One-site-herd: piglet unit production
J	AMO, CLI	*P. multocida, G. parasuis*	Farrow-to-finish: piglet to hog-finished production

Molecular diagnosis

P. multocida was detected in 6 out of 10 farms and *G. parasuis* in 4 out of 10 farms. *G. parasuis* and *P. multocida* were detected together in one sow. *A. pleuropneumoniae* was detected only in farm J. *M. hyopneumoniae* (Mhyo) was not detected in any of the 50 samples. Farm C had Mhyo-free status, but we cannot exclude the presence of *M. hyopneumoniae* in the other farms that tested negative by nested PCR. The agreement between PCR and culture was found in farms A, B, C, H, I, J *for P. multocida. G. parasuis* and *A. pleuropneumoniae* were only detected by PCR, since

they cannot grow on blood agar plates, but *A. pleuropneumoniae* was detected in the slaughterhouse in the herd from farm J. Lung lesions caused by *P. multocida* were reported by the slaughterhouse in the herds from farms B, D, G. We found a positive association between the reduction of lesions at the slaughterhouse and the use of autogenous *P. multocida* vaccines (Fisher Exact Probability Test, P=0.048). As shown in Table 4, six of the 10 farms used autogenous *P. multocida* vaccine and only one of them, farm J, reported lung lesions at slaughter (although isolation showed *A. pleuropneumoniae* in the lesions).

Table 4 - Corresponding diagnostic test by molecular assay and isolate cultured associated lung lesion report.

Farm	PCR	Culture	Lung Lesion
A	*P. multocida*	*A. suis; P. multocida; S. suis.*	No
B	*P. multocida; G. parasuis*	*A. suis; P. multocida; S. suis.*	Yes
C	*G. parasuis*	*A. suis; S. suis*	No
D	*P. multocida*	*B. bronchiseptica*	Yes
E	*P. multocida*	*A. suis; B. bronchiseptica; P. multocida*	Yes
F	*G. parasuis*	*B. bronchiseptica*	No
G	*P. multocida*	*A. suis; B. bronchiseptica; P. multocida; S. suis*	Yes
H	*P. multocida*	*A. suis; P. multocida*	No
I	*G. parasuis*	*A. suis; S. suis*	No
J	*A. pleuropneumoniae*	*S.* ser. Typhimurium*; S. aureus*	Yes

Isolates from the sows` nasal microbiota and resistance profile.

A total of 132 strains belonging to 20 bacterial species were isolated from the 50 sows. *Staphylococcus* coagulase negative, typical commensals of the nasal microbiota was the most prevalent (22/50; 44%). Although Enterobacteriaceae are commonly associated with the gut microbiota, herein *Klebsiella, Pantoea, Proteus, Yersinia, Escherichia*, and *Salmonella* were isolated from the sow nasal swabs. Pathogens such *as P. multocida, B. bronchiseptica, K. pneumoniae, P. aeruginosa, S. aureus, Y. enterocolitica, S.* Typhimurium and *S. suis* were isolated from sows without respiratory diseases. But, as expected, *A. pleuropneumoniae* was isolated from the sow with fever from farm J.

To investigate antimicrobial resistance in the isolates, 23 antimicrobial agents were performed on the 132 isolates (Supplementary Table S1). Overall, the results showed 55% (1573/2840) antimicrobial resistance testing (Supplementary Table S2). The phenotypic profile of antimicrobial resistance varied from 39% to 83% among bacterial species (Supplementary Table S3 and Figure 1) and from 47% to 65% among farms (Supplementary Table S3 and Figure 2). We observed differences between species and farms. For example, the two *A. suis* isolates from farm B showed 100% and 63% antimicrobial resistance, respectively, and just considering the beta lactams, *A. suis* strains from the different farms differed in their susceptibility to ampicillin, cephalothin and ceftiofur. Although less spread among the farms, *S.* Typhimurium (isolated only from Farms I and J) and *P. aeruginosa* (isolated only from Farm G) presented the highest percentage of resistance. On the other hand, one *Proteus vulgaris* from farm B and one from farm H were 100% sensible. Notably, *Staphylococcus* coagulase negative showed wide diversity in the number of resistances: the isolate from farm A showed antimicrobial resistance of 74% (17/23); in farm E, 2 isolates showed no resistances, one isolate 8.7% (2/23) and the fourth isolate 43.5% (10/23); isolates from farm F showed 52% (12/23) and 35% (8/23) of antimicrobial resistance; isolates from farm G showed resistance varying from 17.4% (4/23) to 82.6% (19/23).

Considering the history of use of each antibiotic reported, the longest reported period of antibiotic use was nine years for bacitracin and the shortest was one year for amoxicillin with clavulanic acid, marbofloxacin, and gentamicin. Bacitracin had the highest resistance (93.9%), followed by florfenicol (76.5%). We also observed lower resistance to amikacin, which was never used in the herds.

It is also noteworthy to highlight the presence of resistance to ceftiofur (a third-generation cephalosporin) in several taxa, including *A. suis, S. aureus* and *S. suis.*

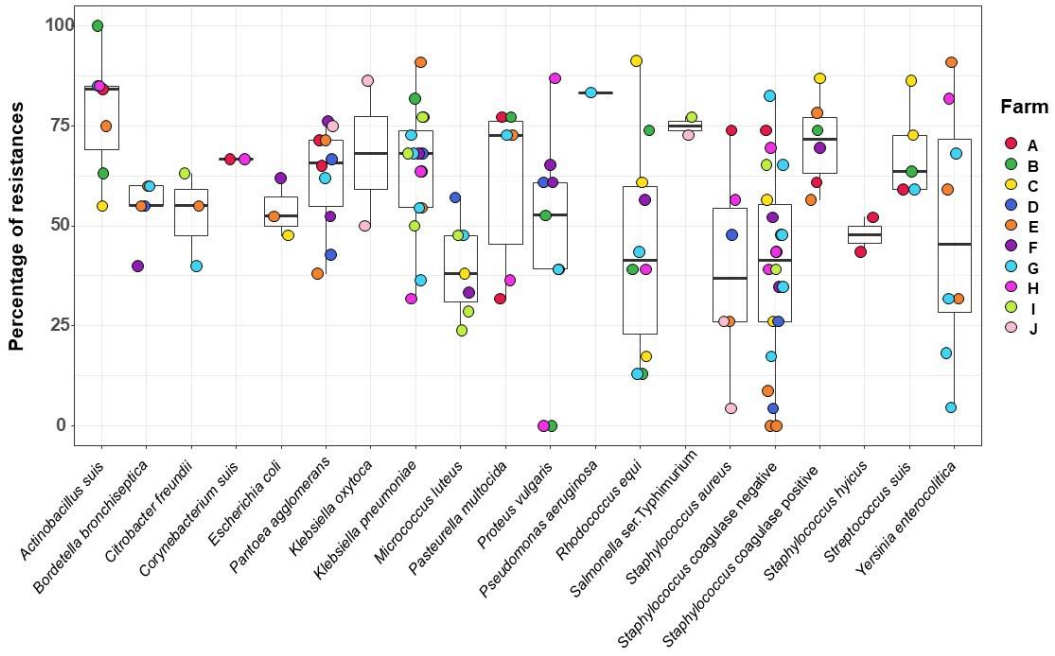

Figure 1 - Percentage of antimicrobial resistance per bacterial species

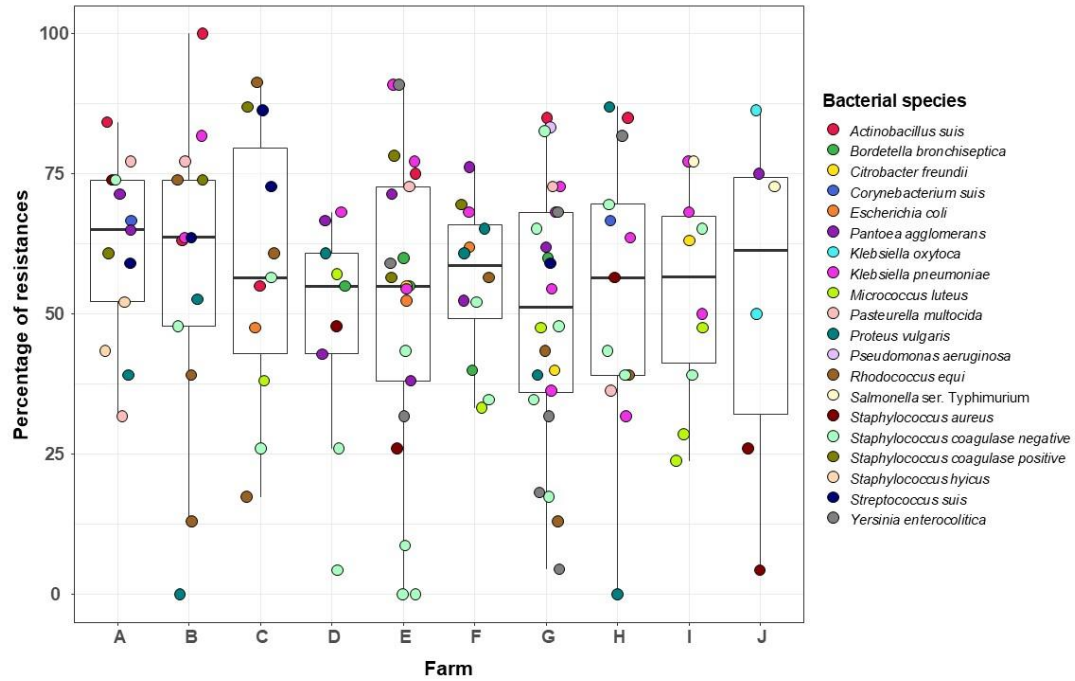

Figure 2 - Percentage of antimicrobial resistance per farm

When the resistance to different antibiotic families was examined, the majority of isolates showed multiresistance to 7-9 antibiotic classes (Supplementary Table S2; Figure 3). On the other hand, only 5 isolates did not show any resistance (2 *Proteus* and 3 *Staphylococcus*), 2 isolates showed resistance to just one antibiotic family, and one isolate to 2 families.

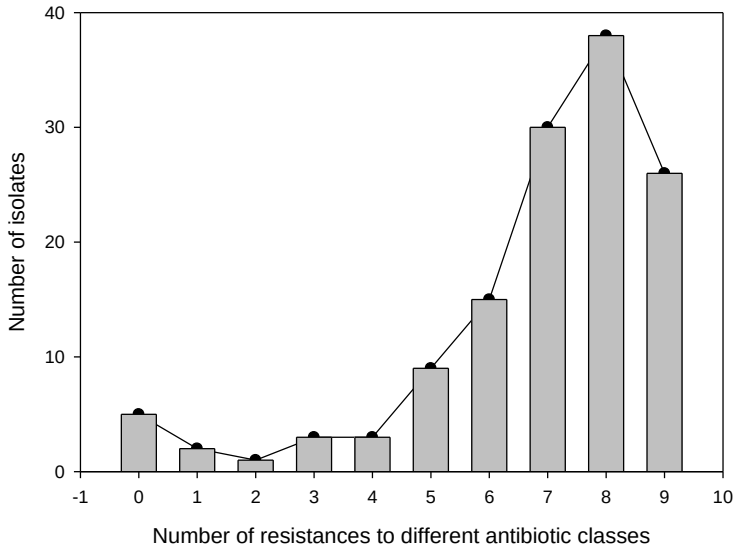

Figure 3 – Number of resistances to different antibiotic class

Discussion

This cross-sectional study evaluates, for the first time, the antimicrobial resistance of species collected from the nasal cavities of sows in the Federal District of Brazil. We screened for the most common respiratory pathogens in swine production (7,8;12-14;18-20). In general, a high level of antimicrobial resistance was observed in most of the isolates, especially in *P. aeruginosa, A. suis* and *S.* Typhimurium. Unexpectedly, the antimicrobial resistance was not associated with the biosecurity score. Consistent with a previous report, this score did not correlate with antimicrobial use [34].

Noteworthy, we found *S. suis, A. suis, S. aureus* isolates that were resistant to ceftiofur. In Spain, Blanco-Fuertes et al. [15] observed treatment with ceftiofur administered to sows resulted in higher levels of resistance genes in weaned piglets. Ceftiofur administration had a longer effect on the nasal microbiota composition of piglets when administered to their sows before farrowing than when applied directly to the piglets at birth. In addition, ceftiofur treatment alone, either in sows or piglets, did not improve piglet health or productivity [35]. The finding of *S. suis, A. suis, S. aureus* cephalosporin-resistant is concerning since third and fourth-generation cephalosporins are considered critically important antibiotics in human medicine [36]. In addition, resistance to ceftiofur has been described in *P. multocida* from cattle origin [37], and in a *P. multocida* isolate of wildlife origin [38], reinforcing the fact that AMR is a multifactorial problem, with intrinsic links in the human, animal and environmental interface. Furthermore, *Pasteurellaceae* isolates from wild

and domestic animals in an alpine ecosystem in northeastern Spain exhibited similar levels of resistance for macrolides [38].

This research also found a high level of resistance to macrolides, particularly clindamycin and tylosin. Clindamycin was used in the medicated feed for sows on farms A and J, and tylosin was used in the medicated feed for sows on farm I. Curiously, most isolates from farms A and J were no longer susceptible to clindamycin, and all isolates from farm I showed resistance to tylosin.

In addition to resistance, antimicrobials could be a significant cause of dysbiosis in the offspring. In this way, Bonillo-Lopez et al. [40] showed that sow treatment reduces the nasal bacterial load of sows and alters the composition of the nasal microbiota of piglets, showing unusual taxa microbiota. But not only the antimicrobials applied by intramuscular injection induce deleterious effect on the microbiota. Mou et al. [39] found that oral oxytetracycline had a greater effect on the diversity and disruption of the microbiota than the intramuscular route. They described different dosing regimens of oxytetracycline associated with shifts in the nasal microbiota of their offspring [38].

Colonization by respiratory pathogens not always leads to disease, since mechanisms involved in the early local innate immune response might favor colonization without clinical illness development [41]. Still, some farm managers in this survey alternated penicillin, florfenicol, tylosin, clindamycin, tetracycline, enrofloxacin and oxytetracycline with amoxicillin in the feed, disregarding that metaphylactic treatments can be avoided without negatively impacting the production [15]. Medicated feed is still a common strategy in many countries to control the occurrence of *Mycoplasma, Pasteurella, Glaesserella* [9] and *S. suis* [42].

S. suis, an emerging zoonotic pathogen [43] widely distributed in pig farms [44] was isolated in farms A, B, C, and G, and presented antimicrobial resistance. Being a ubiquitous component of the microbiota of the upper respiratory tract [45] pigs are usually colonized by more than one serotype, but only a few strains can induce disease [46]. Recent reports found higher levels of resistances in *S. suis* strains isolated from clinically healthy sows in China for tetracycline (91.7%), sulfametoxazol (86.7%), erythromycin (67.2%), and trimethoprim/sulfamethoxazole (59.1%) [20]. Also, *S. suis* isolates from Australia showed high resistance frequencies for tetracycline (99.3%) and erythromycin (83.8%) [46]. On the other hand, *S. suis* resistance to florfenicol (76.5%) and clindamycin (74.2%) was higher in this study than previously reported [20, 47]. It is well known that it is essential to control the spread of pathogenic lineages of *S. suis* through pig populations [45], yet, controlling the transmission of *S. suis* is a challenge. Farms A-J used metaphylactic amoxicillin for sows, but astonishingly, most *S. suis* isolated from the nasal swabs were sensible to amoxicillin. This finding agrees with the statement that the majority of clinical S. suis remains

sensitive to this antibiotic [47]. However, beta-lactam resistant strains are primarily found in commensal sites [42].

Interestingly, there are significant antimicrobial resistances not only about pathogens species, but also among commensals. In line with recent reports, we found *Staphylococcus* coagulase negative, the most prevalent commensal nasal microbiota´s inhabitant, resistant to amoxicillin and penicillin [42, 47]. Brazil has been already characterized *Staphylococcus* coagulase-negative resistant to ampicillin, penicillin and multi-resistance profiles involved in subclinical mastitis [48]. Also, in China, a meta-analysis study was conducted to investigate the epidemiology and antimicrobial resistance rates of *Staphylococcus* coagulase-negative, associated with bovine mastitis, and found the majority to be resistant to beta-lactams [49].

In that regard, the level of use of antimicrobials correlates to the level of resistance toward [50]. Notably, overall resistance was related to the exposition period. Foreseeable, those antimicrobials most used in the sow herd presented higher resistance frequency, except bacitracin. Bacitracin used to be the most often growth promoter chosen and for the longest periods. Since 2018, Bacitracin has been banned in Brazil for use as performance-enhancing additives in food-producing animals [25-26], later than in the European Union [51]. Remarkably, even it is no longer in use, it still presented the highest antimicrobial resistance. Plausibly, this finding could be argued by the vertical transmission of bacterial resistance genes in integrated systems. Some genes can disseminate through the microbial population, leading to the stabilization of the resistance between generations [52]. The presence of resistance genes against the main antimicrobials used highlights the importance of AST to control bacterial diseases that limit herd production [53].

Surprisingly, in this study, *Enterobacteriaceae, Escherichia, Klebsiella, Pantoea, Proteus, Salmonella* and *Yersinia*, were isolated from the sow nasal swabs. *Enterobacteriaceae* family members, together with other gut species, have been demonstrated to be active in the pig nasal microbiota [54] and are likely to play specific roles in the upper respiratory tract, since diversity in the community is crucial in immunity [35, 54]. Therefore, characterizing the composition of the nasal microbiota, in addition to detecting possible resistance genes in the respiratory microbiota [55, 56], can help understand the role and beneficial interactions within the members of the nasal community [15].

Although interactions between bacterial species are not clear, members of the nasal microbiota may be involved in protecting against diseases by preventing colonization by pathogens [41]. Indeed, *G. parasuis* establishes a deferential network involving complex interactions. Mahmmod et al. [56] estimated statistic association for *G. parasuis* colonization, where *Bacteroidaceae*, and *Mycoplasmataceae* in the nasal mucosa of piglets were likely to prevent

virulent *G. parasuis* colonization, whereas *Chitinophagaceae* and *Streptococcaceae* were associated with a higher likelihood of colonization by virulent *G. parasuis*. Similarly, pig carriers or non-carriers of *S. aureus* presented a distinct nasal microbiome [58].

Given its importance, the commensal microbiota should not act as a reservoir of resistance genes [59]. In this case, testing commensal communities can be a tool to control the situation. While bacterial isolation and AST are time-consuming and may not be suitable for use in current farm practices [29], multiplex PCR [28] has the potential to be a faster technique implemented for a national antimicrobial resistance surveillance program [60]. In any case, AST [30-31] will help the farm manager to choose the appropriate antibiotic in the event of disease outbreaks [61].

Conclusions

In this study, the sow´s nasal community showed high prevalence of antimicrobial resistance. Future studies regarding implementation of a national antimicrobial resistance surveillance program, concerning the reduction of antimicrobials in food animal producing, is necessary and may include microbiota and pathogen analysis.

Supplementary Materials
The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Table: S1, S2, S3.

Author Contributions
Conceptualization, S.P., and LR.; methodology, S.P., and LR.; software, B.D., and P.O-G; validation, M.R., B.D, and R.F.; formal analysis, F.S.; investigation, S.P., and LR.; resources, S.P. and LR; data curation, R.F.; writing—original draft preparation, L.R.; writing—review and editing, V.A, visualization and editing, F.S.; supervision, S.P.; project administration, S.P and F.S.; funding acquisition, F.S,. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Conflicts of Interest
The authors declare no conflicts of interest.

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CHAPTER III- IRTA-CReSA EXPERIMENT

Introduction

Framework: IRTA/CReSA

This project was financially supported by CAPES, allowing a sandwich PhD between UnB, Brazil (Universidade de Brasília) and IRTA/CReSA, Spain (Centre de Recerca en Sanitat Animal). CReSA was chosen because it is the state-of-the-art research center located on the Campus of UAB (Universitat Autónoma de Barcelona). This center was founded in 1999 and was integrated in IRTA (Institut de Recerca i Tecnologia Agroalimentàries) since 2015. Nowadays, CReSA is the Animal Health program of IRTA, a research institution for agriculture and food research and technology from the Generalitat of Catalonia. The main objective of CReSA is the development of research and technology for animal health. For this purpose, CReSA collaborates with UAB, private companies and other international institutions. CReSA transfers the scientific innovations to the animal production sector, gives advice and technological support to agrifood companies and the public administration, and organizes scientific and technical training programs. This study was carried out with Dr. Lourdes Migura collaboration, and it was supervised by Dr. Virginia Aragón, principal investigator of the research line of swine respiratory microbiota.

Antimicrobial (AMB) Effect on Swine Microbiota

The set of microorganisms that colonize the mucosal surface is referred to as microbiota (Pickard et al. 2017). These microbial communities have different roles and can influence the development of both innate and adaptive branches of the immune system (Günther et al. 2016). A major function of these symbiotic microorganisms is protection against pathogen colonization and overgrowth of indigenous pathobionts (Caballero-Flores et al. 2023) by inhibiting their growth, modifying the microenvironment, or competing for host surfaces (Pirolo et al. 2021).

In fact, AMBs impact the composition of the microbiome and can shift the prevalence of species (Bonillo-Lopez et al. 20-23), and the reduction of commensal species affects microbial activity and homeostasis (Brestoff & Artis, 2013), thus unbalancing the microenvironment (Collignon & McEwen, 2019). Research on alternative trends to enhance the beneficial effects of commensal microbiota is advancing, and to increase knowledge on this topic, studies are underway to select Gram-negative strains with probiotic properties that inhibit pathogen colonization (Correa-Fiz et al. 2016; Lorenzo de Arriba et al. 2018; Lopez-Serrano et al. 2020; Blanco-Fuertes et al. 2023).

Actually, the respiratory microbiota deserves further investigation (Correa-Fiz et al. 2016), the mucosal epithelium of the nasal cavity (Obregon-Gutierrez et al. 2024) is less studied than the gut (Pickard et al. 2017). It is known nasal microbiota colonization of piglets occur very early in life, (Blanco-Fuertes et al. 2023) and later the microbial structure of the upper respiratory tract undergoes a remarkable evolution after birth and tends to stabilize around weaning (Pirolo et al. 2021). Thus, contact with the sow is especially important (Obregon-Gutierrez et al. 2021). Hence, AMB treatment disrupts the nasal microbiota (Bonillo-Lopez et al. 2023), and when it is administered to pregnant sows has a greater effect than when it is administered directly to piglets (Blanco-Fuertes et al. 2023). Specially, double antibiotic treatment increased the deleterious effect on microbiota diversity by reducing some bacteria commonly found in the nasal cavity of piglets (Bonillo-Lopez et al. 2023).

In a matter of fact, the natural source of microorganisms that can shape a healthy nasal microbiota for piglets is in the dams (Blanco-Fuertes et al. 2023) and the balanced microbiome can prevent diseases during the rearing period, such as Glässer's disease (Correa-Fiz et al. 2016). Non-virulent strains of *Glaesserella parasuis* capable of biofilm formation colonize the upper respiratory tract (Bello-Ortí et al. 2014) and protect against virulent strains of *G. parasuis*, preventing the development of polyserositis and arthritis (Brockmeier et al. 2013).

Therefore, the use of AMB in swine herds is of concern (Rigueira & Perecmanis 2024) due to the effect on all microbial communities (Zeon & Kibe 2023) and AMR increase. Recent studies in Spain on bacterial isolates from pigs have evaluated AST of pathogens involving in PRDC (Haimi-Hakala et al. 2017; Vilaró et al. 2023), and also methicillin-resistant *S. aureus* in pigs (Golob et al. 2022; Abdullahi et al. 2023). On the other hand, Lindon et al. (2024) suggest that attempts to study the microbiome should focus on commensals that can provide robust inhibition of both wild-type and resistant strains as an alternative to AMU. Tetracyclines class is the most used AMB in many countries in Europe such as Spain, France, Denmark, Germany and Austria. On the other hand, lincosamide represented 71.9% of AMU in fattening farms from Austria. The use of macrolides was also reported in pig farms located in France (Lekagul et al. 2019). However, much remains to be understood about the relationship between AMR and AMR, especially in the commensal communities of the respiratory tract of healthy pigs (Argudín et al. 2015).

Restrictive control on antimicrobial use in Europe

Currently, policy makers in the European Union (EU) have developed legislation to monitor and regulate exhaustively the AMU in animals, with special focus on livestock (EU 2019/6; EU 2022/1255, Schmerold et al. 2023). In 2003, the European Union published Directive 2003/99/EC of

17 November 2003 on the monitoring of zoonoses and zoonotic agents, which established that Member States should monitor certain zoonotic and commensal bacteria and their associated resistances in their territory, in order to evaluate trends and sources of bacterial antimicrobial resistance. The Ministry for Agriculture, Fisheries and Food monitors resistance in veterinary pathogens involved in zoonoses and by its data system, PRESVET, it is possible to control veterinary prescriptions and collect information on the sales of antimicrobials. Veterinary prescriptions seek the European Medicine Agency (EMA) recommendation, since different AMB families do not have the same risk of generating AMR from a one-health point of view (Vilaró et al. 2020). In Spain, although the National Antibiotic Resistance Plan 2022-2024 (PRAN) doesn´t monitor veterinary pathogens, it communicates for good use of antimicrobials in production aligned with One Health framework. In Catalunya, the conscious use of antimicrobials is involved in the culture of European veterinarians and stockholders. Pig farms are responsible for diagnosing pathogens when they have outbreaks on their farms. Normally the veterinarian prescribes, but he takes a sample and sends it to the laboratory to make sure that what he has prescribed is correct. And once he has the results of the diagnosis, he checks that he has medicated correctly. The pig producers' initiative has enabled an epidemiological approach to carry out the prudent use of antimicrobials in pigs for respiratory pathogens on a large scale in Spain. Thus, by compiling each clinical case, it is possible to provide pig farmers with a practical approach to antimicrobial susceptibility as well as the prioritization proposed by the EMA, which divides the AMBs into four categories, from A (avoid), B (restrict), C (caution) to D (prudence). Therefore, the selection of an AMB to treat a clinical case must begin with drugs belonging to category D and then with AMBs from category C and B, respectively, if the treatment fails to cope with the bacterial infection (Vilaró et al. 2020).

The current EU legislation regarding antimicrobials (EU 2019/6) have focused special attention to restrict as much as possible the use of AMB, but, if necessary, to prioritize the use of some AMB families versus others in animals following the recommendations addressed by the EMA in 2019 (EU 2019/6). ‘One Health’ approach is recommended for continued AMR monitoring (WHO, 2015) preferably using the broth microdilution test with easy read points. Determining the MIC of an AMB agent is the first step in estimating epidemiological cut off values (ECOFFs). ECOFFs are used to define the upper end of the wild-type distribution of MIC values to distinguish between organisms that are susceptible to a drug and those that are resistant (EU 2019/6). Determining the MIC of an AMB agent is the first step in estimating epidemiological cut off values (ECOFFs).

Therefore, the aim of this study was to evaluate the AMR phenotype of bacterial species associated with sow´s mucous membrane, piglet nasal surface and colostrum in apparently healthy

sows. Those isolates were already disponible in the laboratory. This study contributes to the knowledge on AMR circulating strains which can threaten the successful treatment of bacterial infections in animals and humans.

Materials and Methods

Samples

This study is part of a bigger research about swine nasal microbiota named the CORES project. The CORES project aims to observe the influences exerted on the nasal mucosa of newborn piglets and to identify which microbiota have the greatest impact on the establishment of the nasal mucosa of the litter. The CORES project will investigate whether microorganisms from the vaginal mucosa colonize the upper respiratory tract of piglets during passage through the birth canal; how much colostrum contributes to the formation of the nasal microbiota during the first feeds and last, if the nasal microbiota of the newborn piglets is though more like the nasal microbiota of their mothers.

Herein the aim is to characterize the phenotypic resistance profile in these isolates obtained from vaginal swab, colostrum and nasal swab samples of sows and their litter isolated from the first farm sampled in the Cores project. The isolates were already available at the laboratory and samples were obtained as described by Lorenzo et al. 2018 and López-Serrano et al. 2020. *Glaesserella parasuis* was not MIC evaluated due to the impossibility for this bacterium to grow with the microdilution technique (Vilaró et al. 2020). The collection of strains selected for phenotype analysis were: *nasimurium* (n=24), *Moraxella pluranimalium* (n=9), *Actinobacillus rossii* (n=5), *Streptococcus dysgalactiae* (n=4), *Streptococcus suis* (n=3), *Pasteurella multocida* (n=2), *Staphylococcus aureus* (n=5), *Staphylococcus pseudoepidermidis* (n=2) and *Staphylococcus haemolyticus* (n=2).

Susceptibility testing

Susceptibility testing was performed by broth microdilution using commercial plates (Sensititre®) and following the European Committee on AST by EUCAST guidelines. MIC was defined as the lowest concentration of the AMB that prevents the growth of the targeted bacteria. To define the wild-type (hereafter susceptible) and non-wild type population (hereafter resistant), we used epidemiological cut-off values (ECOFF) when available. A MIC value below the ECOFF indicates that the bacterium is likely from a wild-type drug-susceptible population. Conversely, a MIC value above the ECOFF indicates that the bacterium is likely to be resistant. A wild-type strain is a bacterial strain that is selected as a reference and is well characterized. It is the most common phenotype in a natural population. On the other hand, a non-wild-type strain is a bacterial organism

that has mutations or resistance genes. Mutants may develop the ability to grow without a certain nutrient or the ability to grow in the presence of a toxic substance. From published articles, pharmaceutical industry drug development, resistance monitoring plan, and individual laboratory programs, we can identify "breakpoints" established by the EUCAST or CLSI.

To evaluate AMR of the selected strains, we based on ECOFFs available. However, there wasn´t ECOFFs published for all bacterial species of this study, thus we have to extrapolated some of them from other bacteria closely related, and/or AMB agent belonging to the same AMB class. The MIC test was performed as guide´s instructions SENSITRE^{TM} GRAM-POSITIVE PLATE FORMAT code: GPN3F and GRAM-NEGATIVE PLATE FORMAT plate code: BOPO6F.

The MIC is recorded as the lowest concentration of AMB that inhibits visible growth. The positive controls were *S. aureus* for Gram-positive and *E. coli* for Gram-negative bacteria. Mueller-Hinton medium was used for *P. multocida* and *Staphylococcus* spp. and Blood Mueller-Hinton for *R. nasimurium, M. pluranimalium, Streptococcus* spp. and *A. rossi*. Fresh colonies were used and tested in 96-well commercial Gram-negative plate format BOPO6 and Gram-positive plate format GPN3F to determine the MIC by dilution.

For each strain, a 0.5 McFarland suspension was prepared in saline solution. For *R. nasimurium, M. pluranimalium, A. rossi, S. dysgalactiae* and *S. suis*, 10 µL of the McFarland suspension were pipetted into 10 ml of Blood Mueller Hinton, while 10 µL of the McFarland suspension of *P. multocida, S. aureus, S. haemolyticus, S. pseudoepidermidis* and *S. chromogenes* were pipetted into 10 ml of Mueller Hinton. Finally, 50 ul of the respective microorganism solution was individually pipetted in 96-well plates wells except the negative control. The bacterial concentration was approximately 10^5 CFUs. Plates were incubated overnight, or up to 48 hours for *M. pluranimalium*, at 37ºC with 5% CO2. Results were read manually by visual reading of growth. So, after incubation, each plate was exposed to light and read under a magnifying glass. The control wells for growth were read first. Growth appears as a deposit of cells at the bottom of a well. The MIC values are taken as the first concentration of the AMB that inhibits visible growth as presented in Figure 1.

a) Mueller Hinton (MH) MIC test b) Blood Mueller Hinton (BMH) MIC test

Figure 1 – Lecture of MH and BMH plate format by MIC test

R. nasimurium, M. pluranimalium, A. rossi, S. dysgalactiae, S. suis, P. multocida were tested for the following AMBs: Ampicilin (AMP 0.12-16 µg/mL); Ceftiofur (XNL 0.25-8 µg/mL); Chlortetracycline (CTET 0.5-8 µg/mL); Clindamycin (CLI 0.25-16 µg/mL); Danofloxacin (DANO 0.12-1 µg/mL); Enrofloxacin (ENRO 0.12-2 µg/mL); Florfenicol (FFN 0.25-8 µg/mL); Gentamicin (GEN 1-16 µg/mL); Neomycin (NEO 4-32 µg/mL); Oxytetracycline (OXY 0.5-8µg/mL); Penicillin (PEN 0.12-8 µg/mL); Spectinomycin (SPE 8-64 µg/mL); Sulfadimetoxin (SDM µg/mL); Thrimetroprim/sulfamethoxale (SXT 2/38 µg/mL); Thulathromycin (TUL 1-64 µg/mL); Tiamulin (TIA 1-32 µg/mL); Tilmicosin (TIL 4-64 µg/mL) and Tylosin (TYLT 0.5-32 µg/mL).

Staphylococcus spp. were tested against Ampicillin (AMP 0.5-16 µg/mL); Ceftriaxone (AXO 8-64 µg/mL); Ciprofloxacin (CIP 0.5-1 µg/mL); Clindamycin (CLI 0.12-2 µg/mL); Daptomycin (DAP 0.25-8 µg/mL); Erythromycin (ERY 0.25-4 µg/mL); Gatifloxacin (GAT 1-8 µg/mL); Gentamicin (GEN 500 ng/mL); Levofloxacin (LEVO 0.25-8 µg/mL); Linezolid (LZD 0.5-8 µg/mL); Oxacillin+2%NaCl (OXA+ 0.25-8 µg/mL); Penicillin (PEN 0.06-8 µg/mL); Quinupristin/Dalfopristin (SYN 0.12-4 µg/mL); Rifampin (RIF 0.5-4 µg/mL); Streptomycin (STR 1000 ng/mL); Tetracycline (TET 2-16 µg/mL); Thrimetroprim/Sulfamethoxale (SXT 1/19-4/76 µg/mL); and Vancomycin (VAN 1-128 µg/mL). MIC values based on ECOFF referenced and the means percentage of resistance of each isolate are detailed in Tables 1 – 9.

Results

Susceptibility testing by templates

BOPO6F

Frequencies of MIC values for the different antimicrobials were tabulated separately for each of species sample collection. Table 1 to 6 describe AST of species tested by BOPO6F template.

Table 1 – Number of isolates for 24 strains of *R. nasimurium* with MIC (µg/ml) of respectively AMB concentrations and means percentage of resistance for ECOFF extrapolated to *Staphylococci*.

AMB	≤0.12	0.25	0.5	1	2	4	8	16	32	64	>128	ECOFF	% R
AMP		≤22	0	2	0	0	0	0				0.5	8.3
XNL		0	0	2	12	7	≥3					2	45
PEN	≤15	7	0	0	1	1	0					0.5	8.3
GEN			18	2	4	0	0	0				2	0
NEO				≤24	0	0	0	0	0			1	0
SPE							≥18	1	1	1	≥3	128	0
DANO	0	0	5	≥19								2	0
ENRO	0	0	12	10	≥2							0.5	50
SDM									0	0	>24	NR	X
SXT	≤20	4										0.25	0
FFN		0	1	11	10	2	0					8	0
TYLT			≤23	0	0	0	0	0	1	0		4	4
TIA				≤1	11	12	0	0	0			2	50
TUL				0	1	7	6	8	2	0		8	42
TIL						24	0	0	0	0		4	0
CLI		0	0	0	20	4	0	0				0.5	100
CTET			≤16	2	3	3	0					0.5	42
OXY			≤11	3	2	3	5					0.5	54

* Number of isolates with MIC (µg/ml) of respectively antimicrobial concentrations; ECOFF indicate breakpoints for resistance. NR: Non-reference; X – not possible to estimate; % R means percentage of resistance. | : Lines indicate breakpoint for resistance when available; (<): minimum value of concentration used; (>): maximum value of concentration used. Abbreviations AMB (antimicrobial agents): AMP:Ampicilin; XNL:Ceftiofur; PEN:Penicilin; GEN:Gentamicin; NEO:Neomycin; SXT:Spectinomycin;DANO:Danofloxacin; ENRO:Enrofloxacin; SDM:Sulfadimetoxin; SXT:Trimethropim/Sulfamethoxazole; FFN:Florfenicol; TYLT:Tylosin tartrate; TIA:Tiamulin; TUL:Tulathromycin; TIL:Tilmicosin; CLI:Clindamycin. CTET:Chlortetracycline; OXY:Oxytetracycline.

Table 2 – Number of isolates for 9 strains of *M.plurianimalium* with MIC (µg/ml) of respectively AMB concentrations and means percentage of resistance for extrapolated ECOFF to *M. catarrhalis.*

AMB	≤0.12	0.25	0.5	1	2	4	8	16	32	64	>128	ECOFF	% R
AMP	0	0	1	0	1	4	1	2				0.03	100
XNL	0	3	2	3	1	0	0					0.06	66
PEN	0	0	0	0	0	0	1	8				0.03	100
GEN			7	0	0	1	1	0				NR	X
NEO				0	4	3	0	0	0	0		NR	X
SPE							0	2	2	5	0	NR	X
DANO	0	0	1	8								1	0
ENRO	0	0	0	3	5							1	55
SDM									0	5	≥4	NR	X
SXT	≤5	4										1	X
FFN		<1	8	0	0	0	0					NR	X
TYLT					0	0	5	4	0	0		NR	X
TIA			≤4	3	2	0	0	0	0	0		NR	X
TUL				0	2	1	2	0	3	>1		NR	X
TIL						9	0	0	0	0		NR	X
CLI		0	0	0	3	5	1	0				NR	X
CTET			≤4	2	2	1	0					NR	X
OXY			<2	0	1	2	4					NR	X

* Number of isolates with MIC (µg/ml) of respectively antimicrobial concentrations; ECOFF indicate breakpoints for resistance. NR: Non-reference; X – not possible to estimate; % R means percentage of resistance. | : Lines indicate breakpoint for resistance when available; (<): minimum value of concentration used; (>): maximum value of concentration used. Abbreviations AMB (antimicrobial agents): AMP:Ampicilin; XNL:Ceftiofur; PEN:Penicilin; GEN:Gentamicin; NEO:Neomycin; SXT:Spectinomycin;DANO:Danofloxacin; ENRO:Enrofloxacin; SDM:Sulfadimetoxin; SXT:Trimethropim/Sulfamethoxazole; FFN:Florfenicol; TYLT:Tylosin tartrate; TIA:Tiamulin; TUL:Tulathromycin; TIL:Tilmicosin; CLI:Clindamycin. CTET:Chlortetracycline; OXY:Oxytetracycline.

Table 3 – Number of isolates for 5 strains of *A. rossi* with MIC (µg/ml) of respectively AMB concentrations and means percentage of resistance for extrapolated ECOFF to *A. pleuropneumoniae.*

AMB	≤0.12	0.25	0.5	1	2	4	8	16	32	64	≥128	ECOFF	%R
AMP		≤4	0	1	0	0	0	0				0.5	20
XNL	≤2	0	1	1	0	0	1					0.06	60
PEN	≤4	0	1	0	0	0	0					1	0
GEN			<2	0	1	1	0	1				32	0
NEO				0	2	0	1	1	0	>1		64	0
SPE						1	0	1	1	0	≥2	NR	X
DANO	0	0	3	2	19							NR	X
ENRO	≤2	1	2	0	0							0.125	100
SDM									0	0	≥5	NR	X
SXT	≤5	0										0.25	100
FFN		0	1	0	0	4	0					1	80
TYLT			<2	1	0	1	0	0	1			64	0
TIA		0	0	0	3	0	0	0	2	0		32	0
TUL				0	0	0	1	0	1	>3		64	0
TIL						4	0	0	0	>1		NR	X
CLI		0	0	0	1	2	0	2				NR	X
CTET			<1	1	1	1	1					1	60
OXY			≤4	0	0	1	0					1	20

* Number of isolates with MIC (µg/ml) of respectively antimicrobial concentrations; ECOFF indicate breakpoints for resistance. NR: Non-reference; X – not possible to estimate; % R means percentage of resistance. | : Lines indicate breakpoint for resistance when available; (<): minimum value of concentration used; (>): maximum value of concentration used. Abbreviations AMB (antimicrobial agents): AMP:Ampicilin; XNL:Ceftiofur; PEN:Penicilin; GEN:Gentamicin; NEO:Neomycin; SXT:Spectinomycin;DANO:Danofloxacin; ENRO:Enrofloxacin; SDM:Sulfadimetoxin; SXT:Trimethropim/Sulfamethoxazole; FFN:Florfenicol; TYLT:Tylosin tartrate; TIA:Tiamulin; TUL:Tulathromycin; TIL:Tilmicosin; CLI:Clindamycin. CTET:Chlortetracycline; OXY:Oxytetracycline.

Table 4 – Number of isolates for 4 strains of *S.dysgalactiae* with MIC (µg/ml) of respectively AMB concentrations and means percentage of resistance for ECOFF established for *S.dysgalactiae.*

AMB	≤0.12	0.25	0.5	1	2	4	8	16	32	64	≥128	ECOFF	%R
AMP		3	0	0	0	0	1	0				0.25	25
XNL	0	3	0	1	0	0	0					2	0
PEN	≤1	0	0	0	0	0	3					0,125	75
GEN			1	0	1	2	0	0				8	0
NEO	0	0	0	0	0	1	0	1	0	≥2		128	0
SPE						4	0	0	0	0	0	128	0
DANO	0	0	0	4	19							2	0
ENRO	0	0	0	4	0							NR	X
SDM									0	0	≥4	NR	X
SXT	≤4	0										0.25	100
FFN		0	0	1	3	0	0					4	0
TYLT		0	0	0	0	0	0	1	3			1	100

TIA	0	0	0	0	0	0	0	0	≥4		NR	X
TUL				0	0	0	0	0	≥4		NR	X
TIL				3	0	0	0	0	≥1		NR	X
CLI	0	0	0	0	0	3	1				0.25	100
CTET		≤2	0	0	0	2					0.5	50
OXY		0	2	0	0	2					0.5	100

* Number of isolates with MIC (µg/ml) of respectively antimicrobial concentrations; ECOFF indicate breakpoints for resistance. NR: Non-reference; X – not possible to estimate; % R means percentage of resistance. │: Lines indicate breakpoint for resistance when available; (<): minimum value of concentration used; (>): maximum value of concentration used. Abbreviations AMB (antimicrobial agents): AMP:Ampicilin; XNL:Ceftiofur; PEN:Penicilin; GEN:Gentamicin; NEO:Neomycin; SXT:Spectinomycin;DANO:Danofloxacin; ENRO:Enrofloxacin; SDM:Sulfadimetoxin; SXT:Trimethropim/Sulfamethoxazole; FFN:Florfenicol; TYLT:Tylosin tartrate; TIA:Tiamulin; TUL:Tulathromycin; TIL:Tilmicosin; CLI:Clindamycin. CTET:Chlortetracycline; OXY:Oxytetracycline.

Table 5 – Number of isolates for 3 strains of *S.suis* with MIC (µg/ml) of respectively AMB concentrations and means percentage of resistance for ECOFF established for *.suis* and when necessary, extrapolated ECOFF to *S.dysgalactiae*

AMB	≤0.12	0.25	0.5	1	2	4	8	16	32	64	≥128	ECOFF	%R
AMP		0	1	0	0	0	1	0				0.25	66
XNL	≤3	0	0	0	0	0	0					2	0
PEN	≤1	1	0	1	0	0	0					0.125	66
GEN				2	1	0	0	0				16	0
NEO				0	2	0	0	1	0	0		NR	X
SPE						0	0	0	1	1	>1	128	0
DANO	≤2	0	0	1	19							2	0
ENRO	0	1	0	0	2							NR	X
SDM									0	0	>3	NR	X
SXT	≤3	0										0.25	0
FFN		0	1	1	1	0	0					4	0
TYLT				0	0	0	0	0	3			1	100
TIA				0	0	0	0	1	0	2		NR	X
TUL					0	0	0	0	1	2		NR	X
TIL						0	0	0	1	0	>2	NR	X
CLI		0	0	0	0	0	0	3				0.25	100
CTET			≤1	1	0	0	1					0.5	66
OXY			≤1	1	0	0	1					0.5	66

* Number of isolates with MIC (µg/ml) of respectively antimicrobial concentrations; ECOFF indicate breakpoints for resistance. NR: Non-reference; X – not possible to estimate; % R means percentage of resistance. │: Lines indicate breakpoint for resistance when available; (<): minimum value of concentration used; (>): maximum value of concentration used. Abbreviations AMB (antimicrobial agents): AMP:Ampicilin; XNL:Ceftiofur; PEN:Penicilin; GEN:Gentamicin; NEO:Neomycin; SXT:Spectinomycin;DANO:Danofloxacin; ENRO:Enrofloxacin; SDM:Sulfadimetoxin; SXT:Trimethropim/Sulfamethoxazole; FFN:Florfenicol; TYLT:Tylosin tartrate; TIA:Tiamulin; TUL:Tulathromycin; TIL:Tilmicosin; CLI:Clindamycin. CTET:Chlortetracycline; OXY:Oxytetracycline.

Table 6 – Number of isolates for 2 strains of *P.multocida* with MIC (µg/ml) of respectively AMB concentrations and means percentage of resistance for ECOFF established for *P.multocida*.

AMB	≤0.12	0.25	0.5	1	2	4	8	16	32	64	≥128	ECOFF	%R
AMP		1	0	0	0	0	0	0				0.5	0
XNL	≤1	0	0	1	0	0	0					0.06	50
PEN	≤2	0	0	0	0	0	0					0.5	0
GEN				2	0	0	0	0				8	0

NEO				0	0	2	0	0	0	0		16	0
SPE							>1	0	0	1	0	64	0
DANO	≤1	0	0	1								NR	X
ENRO	≤2	0	0	0	0							0.06	0
SDM									0	0	>2	NR	X
SXT	≤2	0										0.125	0
FFN		0	2	0	0	0	0					1	0
TYLT			1	0	1	0	0	0	0			64	0
TIA		0	0	0	0	0	0	1	0	1		64	0
TUL					1	0	0	1	0	0		8	50
TIL					2	0	0	0	0	0		32	0
CLI		0	0	0	1	0	0	1				8	50
CTET			≤1	1	0	0	0					1	0
OXY			≤1	1	0	0	0					2	0

* Number of isolates with MIC (µg/ml) of respectively antimicrobial concentrations; ECOFF indicate breakpoints for resistance. NR: Non-reference; X – not possible to estimate; % R means percentage of resistance. │: Lines indicate breakpoint for resistance when available; (<): minimum value of concentration used; (>): maximum value of concentration used. Abbreviations AMB (antimicrobial agents): AMP:Ampicilin; XNL:Ceftiofur; PEN:Penicilin; GEN:Gentamicin; NEO:Neomycin; SXT:Spectinomycin;DANO:Danofloxacin; ENRO:Enrofloxacin; SDM:Sulfadimetoxin; SXT:Trimethropim/Sulfamethoxazole; FFN:Florfenicol; TYLT:Tylosin tartrate; TIA:Tiamulin; TUL:Tulathromycin; TIL:Tilmicosin; CLI:Clindamycin. CTET:Chlortetracycline; OXY:Oxytetracycline.

GPN3F

Tables 7 to 9 describe AST of *Staphylococci* performance by GPN3F template

Table 7 – Number of isolates for 5 strains of *S. aureus* with MIC (µg/ml) of respectively AMB concentrations and means percentage of resistance for ECOFF published for *S. aureus*.

AMB	≤0.12	0.25	0.5	1	2	4	8	16	32	64	≥128	ECOFF	% R
AMP		≤3	0	0	0	0	2	0				0.5	20
AXO						5	0	0	0	0		8	0
OXA+	≤3	1	0	0	0	1	0	0				2	20
PEN	≤2	1	0	0	0	2	0	0				0.5	20
GEN			4	0	0	0	0					2	0
STR								<5				16	0
CIP	0	2	0	1	2							2	0
GAT		3	0	0	2	0	0	0				0.25	40
LEVO	≤3	0	0	0	0	0	2	0				1	40
SXT	≤5											0.25	0
ERI	≤3	2	0	0	0	0						1	0
CLI	≤2	1	0	0	2							0.25	40
SYN	≤1	1	2	1	0							1	0
TET				3	1	0	0	>1				1	40
DAP	≤3	1	0	1	0	0	0	0				1	0
VAN			4	0	1	0	0	0	0	0	0	2	0
LZD			0	4	1	0	0	0	0	0	0	4	0
RIF		5	0	0	0	0	0			N	NT	0.03	0

*Number of isolates with MIC (µg/ml) of respectively antimicrobial concentrations; ECOFF indicate breakpoints for resistance; % R means percentage of resistance. │: Lines indicate breakpoint for resistance when available; (<): minimum value of concentration used; (>): maximum value of concentration used. Abbreviations(antimicrobial agents):AMP:Ampicillin; AXO:Ceftriaxone; OXA+:Oxacillin + 2% NaCl; PEN:Penicillin; GEN:Gentamicin;

STR:Streptomycin; CIP: Ciprofloxacin; GAT:Gatifloxacin; LEVO:Levofloxacin;
SXT:Trimethoprim/sulfamethoxazole; ERI:Erythromycin; CLI:Clindamycin; SYN:Quinupristin/dalfopristin;
TET:Tetracycline; DAP:Daptomycin; VAN:Vancomycin; LZD:
Linezolid; RIF:Rifampin

Table 8 – Number of isolates for 2 strains of *S. haemolyticus* with MIC (µg/ml) of respectively AMB concentrations and means percentage of resistance for ECOFF published for *S. haemolyticus* and when necessary, extrapolated ECOFF to *S. aureus.*

AMB	≤0.12	0.25	0.5	1	2	4	8	16	32	64	≥128	ECOFF	% R
AMP	≤1	0	1	0	0	0	0	0				0.5	0
AXO						2	0	0	0	0		8	0
OXA+	≤1	0	1	0	0	0	0	0				0.5	0
PEN	≤1	0	1	0	0	0	0	0				1	0
GEN				1	2	0	0	0	0			2	0
STR								≤2				16	0
CIP		≤1	0	1	0							1	0
GAT		≤2	0	0	0	0	0	0				0.25	0
LEVO	≤1	0	0	1	0	0	1	0				1	0
SXT	≤2											0.25	0
ERI	≤1	0	0	0	0	1						1	50
CLI	0	0	1	1	0	0						0.5	50
SYN	≤1	1	0	0	0							1	0
TET				≤1	0	0	0	>1				1	50
DAP	0	≤1	1	0	0	0	0	0				1	0
VAN			1	1	0	0	0	0	0	0	0	4	0
LZD			1	1	0	0	0	0	0	0	0	2	0
RIF	≤2	0	0	0	0	0	0					0.06	0

*Number of isolates with MIC (µg/ml) of respectively antimicrobial concentrations; ECOFF indicate breakpoints for resistance; % R means percentage of resistance. │: Lines indicate breakpoint for resistance when available; (<): minimum value of concentration used; (>): maximum value of concentration used. Abbreviations(antimicrobial agents):AMP:Ampicillin; AXO:Ceftriaxone; OXA+:Oxacillin + 2% NaCl; PEN:Penicillin; GEN:Gentamicin;
STR:Streptomycin; CIP: Ciprofloxacin; GAT:Gatifloxacin; LEVO:Levofloxacin;
SXT:Trimethoprim/sulfamethoxazole; ERI:Erythromycin; CLI:Clindamycin; SYN:Quinupristin/dalfopristin;
TET:Tetracycline; DAP:Daptomycin; VAN:Vancomycin; LZD:
Linezolid; RIF:Rifampin

Table 9 – Number of isolates for 2 strains of *S. pseudoepidermidis* with MIC (µg/ml) of respectively AMB concentrations and means percentage of resistance for ECOFF published for *S. pseudoepidermidis* and when necessary, extrapolated ECOFF to *S. aureus.*

AMB	≤0.12	0.25	0.5	1	2	4	8	16	32	64	≥128	ECOFF	%R
AMP	0	0	1	0	0	0	0	1				0.5	50
AXO						1	0	0	0	0		8	0
OXA+	≤1	1	0	0	0	0	0	0				0.5	0
PEN	0	0	0	0	1	0	1	0				0.06	100
GEN		≤2	0	0	0	0	0	0	0			0.25	0
STR								≤2				16	0
CIP		0	0	0	2	0	0					1	100
GAT		≤1	0	0	1	0	0	0				0.25	50
LEVO	0	0	0	0	1	0	1	0				0.5	100
SXT	≤2											0.25	0
ERI	0	≤1	0	0	0	1						0.1	50
CLI	0	0	0	0	2	0						0.25	100
SYN	0	≤1	1	0	0	0						1	0
TET				0	0	0	0	>2				1	100
DAP	<2	0	0	0	0	0	0	0				1	0
VAN			2	0	0	0	0	0	0	0	0	4	0
LZD			1	1	0	0	0	0	0	0	0	4	0
RIF	NT	≤2	0	0	0	0	0					0.03	0

*Number of isolates with MIC (µg/ml) of respectively antimicrobial concentrations; ECOFF indicate breakpoints for resistance; % R means percentage of resistance. │: Lines indicate breakpoint for resistance when available; (<): minimum value of concentration used; (>): maximum value of concentration used. Abbreviations(antimicrobial agents):AMP:Ampicillin; AXO:Ceftriaxone; OXA+:Oxacillin + 2% NaCl; PEN:Penicillin; GEN:Gentamicin; STR:Streptomycin; CIP: Ciprofloxacin; GAT:Gatifloxacin; LEVO:Levofloxacin; SXT:Trimethoprim/sulfamethoxazole; ERI:Erythromycin; CLI:Clindamycin; SYN:Quinupristin/dalfopristin; TET:Tetracycline; DAP:Daptomycin; VAN:Vancomycin; LZD: Linezolid; RIF:Rifampin

Antimicrobial resistant (AMR) patterns

The samples origin was 38% (21/56) from piglet nasal swabs, 27% (15/56) from sow nasal swabs, 20% (11/56) from vaginal swab and 16% (9/56) from colostrum. The MDR patterns were distributed in nasal piglet (42%), sow vaginal (42%); sow nasal (8%) and colostrum (8%). In this study, phenotypic AMR was identified in almost all isolates: 21% (12/56) showed resistance to three or more antimicrobial families (MDR). There were 5 MDR patterns detected in 20% (5/24) of *R. nasimurium* isolates; 20% (1/5) of *A. rossi;* 25% (1/4) of *S. dysgalactiae;* 66% (2/3) of *S. suis* while *P. multocida* (0/2) and *M. pluranimalium* (0/9) did not show MDR. Among *Staphylococci spp*., only *S. pseudoepidermidis* (2/2) showed MDR (Table 10).

Table 10 - Antimicrobial resistant (AMR) patterns detected on isolates cultured from Cores project

Strain code/origin sample*	AMR patterns	Nº Isolates[a]
R. nasimurium		
2N1S	[b]AMP-XNL-PEN-ENRO-TYLT-CLI	1
15N1P	[b]AMP-XNL-PEN-ENRO-CLI	1
10N1P; 24N1P; 6C1S	[b]XNL-ENRO-TIA-CLI	3
1N1P,4N1P; 15N2P, 16N1P; 21N2P, 28N2P	XNL-TIA-CLI	6
3N2S; 7N2P; 13N1P	ENRO-TIA-CLI	3
1C1S,1C3S; 20N1P,8C4S; 7N1S; 1N2P,17N2P; 5N1P; 5N3P; 10C1S	CLI	10
M.plurianimalium		
5N3S; 8C2S; 8C5S;11C3S	XNL-PEN-AMP-ENRO	4
11N2S; 13N2S	XNL-PEN-AMP	2
9N2S	PEN- AMP-ENRO	1
1N3S; 8N2S	PEN-AMP	2
A.rossi		
7V1S	[b]XNL-ENRO-CTET-OXY	1
8V2S	XNL-CTET	1
12V3S	XNL	1
3V2S	ENRO	1
1V3S	NONE	1
S. dysgalactiae		
3V1S	[b]AMP-PEN-TYLT-CLI-OXY	1
9V1S, 5N1S	TYLT-CLI-CTET-OXY	2
15N3P	TYLT-CLI-OXY	1
S. suis		
8V1S, 14V2S	[b]AMP-PEN- TYLT-CLI-CTET-OXY	2
12N2S	TYLT-CLI	1
P.multocida		
12N3S	XNL-TUL	1
13N3S	CLI	1
S.aureus		
15N4P, 13N1S	AMP-PEN-TET	2
7V2S, 14N3S	LEVO-CLI	2
2V2S	NONE	1
S.haemoliticus		
13C1C	ERY-TET-CLI	1
23N1P	NONE	1
S.pseudoepidermidis		
9N1P	[b] AMP-PEN-CIP-GAT-LEVO-CLI-TET	1
7V3S	[b] PEN-CIP-LEVO-ERY-CLI-TET	1

* Strain codes: the letter after the first number indicates the isolation site: N, nasal; V, vaginal; and C, colostrum. The last letter indicates the animal: S, sow and P, piglet.

[a] Number of isolates showing respective AMR pattern. [b] Multidrug-resistant patterns exhibited by resistant isolates.

XNL – Ceftiofur; GEN – Gentamicin; FFN – Florfenicol; TIA – Tiamulina; CTET – Chlortetracycline; OXY- Oxytetracycline; PEN -Penicilin; AMP- Ampicilin; DANO-Danofloxacin; NEO – Neomycin; SXT - Trimethropim/Sulfamethoxazole; SPE – Spectinomycin; TYLT -Tylosin tartrate; TUL – Tulathromycin; TIL – Tilmicosin; CLI – Clindamycin; SDM - Sulphadimethoxine; ENRO – Enrofloxacin; ERY - Erythromicina; STR – Streptomycin; SYN - Quinupristin/dalfopristin; DAP – Daptomycin; VAN – Vancomycin; TET – Tetracycline; RIF – Rifampin; LEVO Levofloxacin; LZD Linezolid; PEN Penicilin; CIP - Ciprofloxacin; AXO – Ceftriaxone; GAT – Gatifloxacin; OXA+ -Oxacilin+2%NaCl.

We found the same pattern of AMR among all sources except for nasal swabs from piglets and vaginal swabs from sows. For example, same pattern of AMR was found at nasal piglet and colostrum represented by *R. nasimurium*; sow nasal and nasal piglet by *R. nasimurium* and *S. aureus;* sow nasal and colostrum by *M. plurianimalium;* sow nasal and vaginal by *A. rossi* and *S. dysgalactiae.*

.

Resistance patterns are widespread across bacteria 95% (53/56), remarking commensals as *R. nasimurium*, *M. pluranimalium*. *A. rossi* and *S. pseudoepidermidis*. Macrolide resistance is notable, with contributions mainly from *R. nasimurium*, *S. suis*, and *S. dysgalactiae*. Resistance to β-lactam was demonstrated by *R. nasimurium*, *M. pluranimalium*, *Streptococci*, *Staphylococci* and *P. multocida*. However, *P. multocida* shows minimal resistance patterns across the AMBs tested. Also, minimal resistance, mainly involving *S. suis* and *S. haemolyticus*, was presented by chlortetracycline and oxytetracycline. Likewise, low resistance was exhibited by levofloxacin, ciprofloxacin, gatifloxacin and tulathromycin, which showed very few or no resistance patterns. Finally, the isolates did not show resistance to others antimicrobial agents tested including danofloxacin, florfenicol, spectinomycin sulfadimetoxin, thrimetroprim/sulfamethoxale, tilmicosin, linezolid; oxacillin+2%NaCl, rifampin, streptomycin and vancomycin.

Figure 2a and 2b evidenced a wide range of variability in AMR among bacterial species and across different AMBs

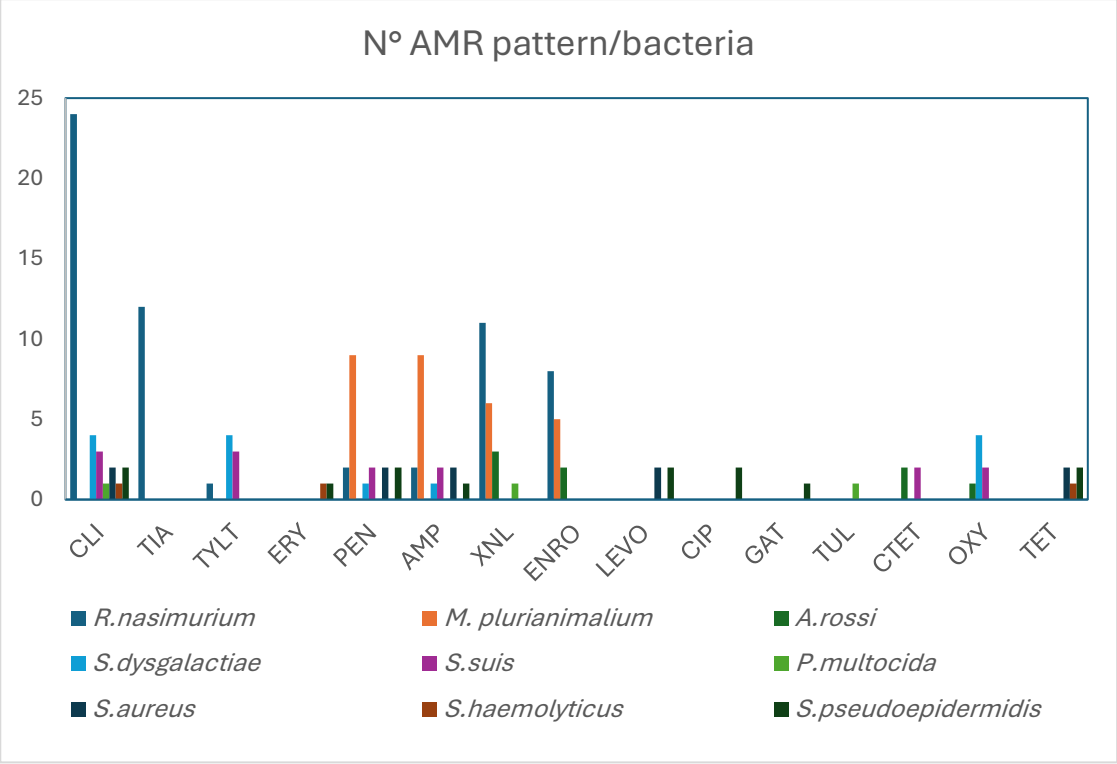

Figure 2a – Number of resistances AMB agent showed on each specie

XNL – Ceftiofur; TIA – Tiamulina; CTET – Chlortetracycline; OXY- Oxytetracycline; PEN -Penicilin; AMP- Ampicilin; TYLT -Tylosin tartrate; TUL – Tulathromycin; CLI – Clindamycin; ENRO – Enrofloxacin; ERY - Erythromicina; TET – Tetracycline; PEN Penicilin; CIP - Ciprofloxacin; GAT – Gatifloxacin.

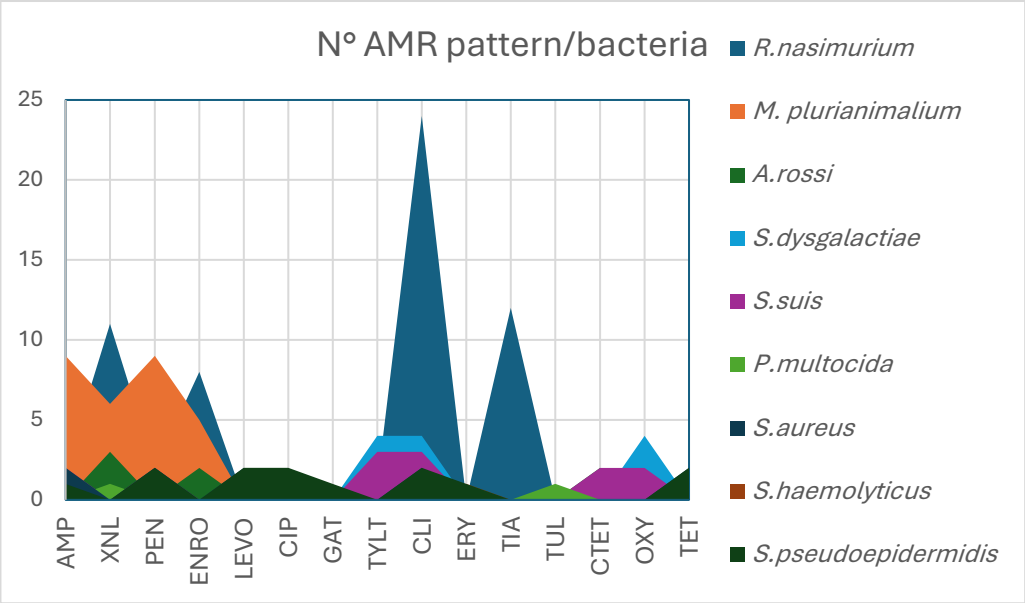

Figure 2b – Number of AMB agents' resistances by AMB agents on all samples
XNL – Ceftiofur; TIA – Tiamulina; CTET – Chlortetracycline; OXY- Oxytetracycline; PEN -Penicilin; AMP- Ampicilin; TYLT - Tylosin tartrate; TUL – Tulathromycin; CLI – Clindamycin; ENRO – Enrofloxacin; ERY - Erythromicina; TET – Tetracycline; PEN Penicilin; CIP - Ciprofloxacin; GAT – Gatifloxacin.

Description by bacterial isolates:

Rothia nasimurium

R. nasimurium is part of swine nasal microbiota and it was isolated from piglets´ nasal swabs 67% (16/24), colostrum 21% (5/24), and sow ´s nasal swabs 12% (3/24). The isolates origin from piglet nasal and colostrum (10N1P; 24N1P; 6C1S) showed the same MDR pattern (XNL-ENRO-TIA-CLI). MIC results were extrapolated to ECOFFs published for *Staphylococcus spp* since there is no ECOFF data available in the literature for *Rothia spp.* Thus, based on extrapolated ECOFF, resistance to clindamycin was exhibited by 100% of *R. nasimurium* strains. Indeed, macrolides class (clindamycin, tiamulin and tylosin tartrate) contributed to 62% (37/60 susceptibility results) of the global resistance among *R. nasimurium* strains, followed by 22% (15/60) of β-lactams (ampicillin, ceftiofur and penicillin) and 13% (8/60) of quinolone class (enrofloxacin).

Moraxella plurianimalium

M. pluranimalium is part of the swine nasal microbiota and it was isolated with more frequency in sow´s nasal cavity 62% (5/8) than piglet´s 38% (3/8). *M. pluranimalium* showed 100% resistance by β-lactams based on ECOFF extrapolated from *M. catarrhalis*, which is known to produce β-lactamases. Penicillin and ampicillin resistance were present in all *M. pluranimalium* strains (9/9), while ceftiofur in 66% (6/9). Half of the isolates (5/9) were also resistant to enrofloxacin.

Actinobacillus rossi

A. rossi was not present on nasal swabs and colostrum, all samples were cultivated from vaginal swab. Based on ECOFF published for *A.pleuropneumoniae* (APP), *A. rossi* isolates showed resistance for ceftiofur 37% (3/8), chlortetracycline 25% (2/8) oxytetracycline 13% (1/8), enrofloxacin 25% (2/8).

Streptococcus dysgalactiae

S dysgalactiae was detected in sow vaginal, sow nasal and piglet nasal swabs. Based on ECOFF extrapolated *to S. agalactiae, S. dysgalactiae* isolates showed 100% resistance to macrolides and to oxytetracycline, while for β-lactams (ampicillin and penicillin) 25%(1/4) resistance were exhibited in two MDR strains from vaginal swab (8V1S and 14V2S).

Streptococcus suis

S. suis was also detected in sow nasal and vagina swabs. *S. suis* isolates showed 100% (3/3) resistance to macrolides (tylosin tartrate and clindamycin). In fact, worldwide, *S. suis* showed resistance to macrolides. It also showed 66% (2/3) for chlortetracycline, while resistance to β-lactams were exhibited in one MDR strain from a vaginal swab (3V1S).

Pasteurella multocida (PM)

Both isolates, detected by sow nasal swabs, showed resistance to macrolides (tulathromycin and clindamycin); while resistance for β-lactams (ceftiofur) was exhibited by one strain. ECOFF for PM and clindamycin was extrapolated to *Mannheimia haemolytica* for erythromycin. Given PM high frequency of involvement in porcine respiratory disease, it is utmost respond quickly with the appropriate AMB in therapy (Truswell et al. 2023). Herein PM did not show MDR pattern.

Staphylococcus aureus

The samples origin from sow nasal (2), sow vaginal (2) and piglet nasal swab (1). *S. aureus* exhibited equally distributed AMR profile, 20% of isolates were resistant to ampicillin, 20% to tetracycline, 20% to levofloxacin, 20% to clindamycin, while 10% for penicillin and 10% for gentamicin.

Staphylococcus haemolyticus

S. haemolyticus origin colostrum and piglet nasal swab While one *S. haemolyticus* strain presented the following pattern: ERY-TET-CLI, the other *S. haemolyticus* strain was pansusceptible.

Staphylococcus pseudoepidermidis

Both isolates of *S. pseudoepidermidis* presented MDR pattern, 9N1P (AMP-PEN-STR-CIP-GAT-LEVO-CLI-TET) and 7V3S (PEN-CIP-LEVO-ERY-CLI-TET). *S. pseudoepidermidis* were detected from piglet nasal and sow vaginal swabs. Little is known about *staphylococci* commensal community, that colonize healthy animals, including their nasal cavities (Abdel-Moein et al. 2022).

Discussion

In Europe, AMU for farmed animals and in aquaculture decreased by around 28% between 2018 and 2022 (EU-27). Even earlier, during 2016 and 2017, AMU for pigs in Denmark was reduced by 5 and 4%, respectively (Holmer et al. 2019). Since 2015, Spain has continued to reduce AMU in livestock, including pigs, by almost 70% (UN 2024) and it has been seen a decline of AMR related to most AMB families in livestock. Vilaró et al. (2023) reported a significant temporal trend ($p < 0.05$) in susceptibility of *Actinobacillus pleuropneumoniae* (APP) for quinolones (enrofloxacin and marbofloxacin), tetracyclines (doxycycline and oxyteracycline), amoxicillin, tiamulin and tilmicosin. Likewise, susceptibility significant temporal trends of *Pasteurella multocida* (PM) were observed for oxytetracycline, tiamulin and sulfamethoxazole/trimethoprim.

Unexpectedly, in this study, we found resistance to macrolides, beta-lactams, and tetracyclines, which are the most used AMBs in the swine industry in Europe (Lekagul et al. 2019), as well in developed countries (Alvarado et al. 2022) and middle-income countries (Cuong et al. 2018). In addition, we only present data on the community profiles of the first pig farm involved in the Cores project, so a single farm is not sufficient to represent the Barcelona pig herds. Besides, the selected farm used AMB prophylactically when castrating male piglets and AMB treatment to control porcine reproductive and respiratory (PRRS) outbreak was reported.

Furthermore, data on resistance is not easy to compare due to the different AST methodologies used along with variate AMB tested (Guitart-Matas et al., 2022). MIC test was applied in this study. Although MIC results may serve as a valid approach (Bovo et al. 2023), the interpretation of most MIC results is challenging due to a lack of ECOFF data (Jong et. al 2013; El Garch et al. 2016; Holmer et al. 2019; Vilaró et al. 2023;). Whereas it is important to evaluate by genetic characterization to correspond the phenotype to the resistance mechanisms, which can be associated with resistance genes (Kehrenberg and Schwarz, 2005; San Millan et al., 2009; Chander et al., 2011; Archambault et al., 2012).

In any case, knowledge of the bacteria susceptibilities is important (Truswell et al. 2023), however, AST results for extrapolated ECOFF should be interpreted with caution, the extrapolated ECOFF may not classify precisely the resistance strains (non-wild type). Interestingly, we have found MDR patterns in commensal such as *R. nasimurium, A. rossi, S. pseudoepidermidis*, while pathogens as *S. aureus* and PM did not. Indeed, PM showed higher susceptibility compared to others bacterial genus. Conversely, Aguilar-Vega et al. (2023) demonstrated more overall AMR for PM than for other pathogens.

Intriguingly, the same AMR pattern was found in distinct origins except for piglet nasal and sow vaginal swabs, suggesting the exchange of resistance genes among these origin strains (Becker et al. 2014; Bonillo-Lopez et al. 2023). Therefore, in this part of the experiment, non-wild strains (AMR profile) colonizing nosocomial newborn piglets´ inhabitants may come from the colostrum or mother´s breathing. Furthermore, the origin and evolution of AMR are involved in complex metabolism processes (Davies & Davies 2010). Moreover, tetracyclines genes were found in fecal samples from piglets in a study by US Agricultural Research Service with no AMB exposure (Looft et al. 2012).

However, AMU in intensive livestock farming is considered an important risk factor for the emergence and spread of resistant bacteria from animals to humans (Albernaz-Gonçalves et al. 2022), that means AMU is one key driver for AMR (Vilaró et al. 2023). Thompson et al. (2023) has described the AMR patterns observed in isolates causing human disease mirror those observed in the locally produced animal food products, suggesting, AMR rates in livestock correlate with AMU policy in each country.

Reported before, in China, the extensive use of tetracycline might have favored the pathogenicity and widespread dissemination of *S. suis* serotype 2, moreover other factors (Zhang et al. 2015). Tetracyclines, including oxytetracycline and doxycycline, are indeed widely used as first-line treatments in livestock. Tetracycline is classified as a Category D drug by the EMA. In Spain, high levels of tetracycline resistance (73.8%) have previously been reported in *A. pleuropneumoniae* (Gutiérrez-Martin et al. 2006). As well, Vilaró et al (2020) found extremely high MIC values for doxycycline for *Bordetella bronchiseptica* in Spain. In fact, tetracycline was reported as the only one of 14 antimicrobials with resistance rate exhibited by PM isolated from pigs around Europe (El Garch et al. 2016). Entering Vetpath, monitoring program for food-producing animals in Europe, observed 82.4% tetracycline resistance in *S. suis* in pigs during the period 2002-2006 (Jong et al. 2023) and 80% during the period 2002-2006 in Spain (Uruén et al. 2024).

Herein, resistance to chlortetracycline and oxytetracycline were found in *S. Suis, S. dysgalactiae* and *A. rossi* and to tetracycline among the *Staphylococcus spp*. Consistent with these findings, in a study of Danish pig farms during the 14-year period from 2004 to 2017, 75% of *S. suis* isolates were found to be resistant to tetracycline (Holmer et al. 2019). As well, tetracycline resistance was also reported in bacterial sampled in Czech Republic (23.9%) (Kucerova et al. 2011), and in Italy (17.2–70%) (Vanni et al. 2012). Besides, marked resistance to tetracycline has been observed in APP isolated from finishing pigs, with APP being the most common cause of acute respiratory outbreaks in Finland (Haimi-Hakala et al. 2017). Also, in a study of ten Belgian pig farms, most *S. epidermidis* (84%) were resistant to tetracycline (Argudín et al. 2015). Similarly, tetracycline

resistance was associated after treatment of porcine respiratory disease in Australia (Dayao et al. 2014). In an update Australian study, Truswell et al. (2023) reported lower AMR of 252 PM isolates (below 1%), except for tetracycline.

On the other hand, tetracycline low susceptibility rates of APP to tetracycline - 0 to 6%- were reported in the United States and Canada between 2011 to 2015 (Sweeney et al. 2017). Also, in Romania (Siteavu et al. 2023) APP reached the highest level in 2020, followed by a decrease in the following year. Instead, in Taiwan (Ke et al. 2024) reported a slight increase of resistance to oxytetracycline and doxycycline during 2017–2022 in 133 isolates of APP isolates but, regarding other AMB classes, Ke et al. (2024) reported highest frequencies for aminoglycosides (streptomycin, kanamycin), β-lactams (ampicillin, amoxicillin), and phenicol (florfenicol) in Taiwan, but not for fluoroquinolones.

Contrarywise Ke et al. (2024), we found resistance to enrofloxacin among *M. plurianimalium A. rossi* isolates and *R. nasimurium.* Supporting our findings, Vilaró et al. (2020) found in APP intermediate resistance to enrofloxacin, but the authors reported decrease resistance of APP in Spain during 2017 to 2019 period. Although quinolones are not active against *Streptococci* because of their intrinsic resistance, fluoroquinolones can be used to treat *Streptococcal* infections (Uruén et al. 2024). Fluoroquinolones are widely used for their broad-spectrum bactericidal activity but should be prescribed carefully to limit resistance development (Siteavu et al. 2023). Also, Siteavu et al. (2023) demonstrate resistance in APP to enrofloxacin and marbofloxacin in Romenia.

Along newer fluoroquinolones, *S. aureus* and *S. pseudoepidermidis* showed resistance to levofloxacin and *S. pseudoepidermidis* also exhibited to gatifloxacin and ciprofloxacin, the last one most used for humans. Though minimal resistance to levofloxacin, ciprofloxacin, and gatifloxacin was observed among *Staphylococcus*, their use in animals is restricted to maintain its effectiveness (EU 2022/1255). The AMBs placed in category B are very highly important in human medicine, thus should only be used when no other AMB options are suitable, and strict measures should be taken to prevent the development of AMR either in pathogens or commensals.

Surprisingly, we found AMR pattern in all isolates of *R. nasimurium*, a commensal of swine nasal microbiota, being 100% resistance to clindamycin. In agreement with this finding, Zhang et al. (2022) isolated *R. nasimurium* from the livers of diseased chickens exhibiting resistance to clindamycin and more than 16 antimicrobials. Clindamycin is used in veterinary medicine to treat major infections caused by Gram-positive bacteria (Abdel-Moein et al. 2022). However, in this study, *S. aureus, S. haemolyticus* and *S. pseudoepidermidis* exhibited clindamycin resistance. Clindamycin resistance is a concern regarding high importance for human medicine, an AMB active ingredient classified as category B (EU 2019/6).

Additionally, *S. dysgalactiae* and *S. suis* exhibited clindamycin and tylosin tartrate resistance in all strains. Pan et al. (2019) described horizontal gene transfer through an integrative conjugative element between *S. suis* strains of different serotypes, helping to explain how MDR is mediated to a wide range of bacteria. Indeed, S. *suis* can act as a reservoir of resistance genes to commensals (Siteavu et al. 2023). Notably, AMR in *S. Suis* has been mostly studied only in diseased animals using surveys that have not evaluated changes over time (Hernandez-Garcia et al. 2017). On the other hand, in a study in England, Hernandez-Garcia reported AMB resistance levels among cases of *S. suis* from diseases cases increased in 2013–2014 relative to 2009–2011 period. Surprisingly, non-clinical *S. suis* isolates were more resistant than *S. suis* isolated from diseased pigs relative to the same period.

In agreement with our findings, Wang et al. (2021) found resistance to clindamycin and lincomycin in all *S. suis* strains isolated from healthy or diseased pigs. These findings agree with Petrocchi Rilo (2024) and Uruén et al (2024), which high AMR rates (>80%) detected for lincosamides in *S. suis* in Spain, and a study review about *S. Suis* infections in pig production by Dechêne-Tempier et al. (2021) underlining resistance to macrolides and tetracyclines. Similarly, Kerdsin et al. (2023) reported all isolated *S. suis* from humans and pigs resistant to clindamycin.

In a matter of fact, macrolides are widely used for the treatment of respiratory tract infections in pigs (Holmer et al. 2019), but resistance to mechanisms to macrolides in *S. suis* was described before (Palmieri et al. 2011; Chen et al. 2013). Resistance pattern for macrolide resistance for *S. Suis* and APP was reported in Danish pig farms for displaying high MIC values to erythromycin, whereas all APP isolates were susceptible to newer macrolide drugs as tulathromycin and tilmicosin (Holmer et al. 2019).

Along with macrolides, β-lactams AMB classes were also tested most resistant in this study. In this study, ampicillin and penicillin resistance was found together in 94% (17/18) of strains. Both resistance pattern was also detected in acute outbreaks of pig respiratory disease in Finland (Haimi-Hakala et al. 2017). Vilaró et al. (2020) found intermediate resistance for amoxicillin in APP in Spain. In this study, amoxicillin hasn´t been tested, but amoxicillin ECOFF was used as referenced to penicillin to *Staphylococci*. The susceptibility to penicillin in our *Staphylococcus* isolates were 44% (4/9), higher than previously reported, whereas *S. epidermidis* isolated in Belgian pig farms were 84% resistance to penicillin (Argudín et al. 2015). Bacteria can be resistant to an AMB because of an intrinsic characteristic of the species (Aguilar-Vega et al. 2023), *Staphylococci* are known for its resistance to β-lactam antibiotics, due to the presence of beta-lactamase enzymes or the mecA gene (Becker et al. 2014).

In this context, *Moraxella* species, particularly *Moraxella catarrhalis*, are also known to produce β-lactamase enzymes, as well as low membrane permeability (Aguilar-Vega et al. 2023),

rendering AMB ineffective (Becker, 2014). It has been demonstrated that the AMR profile of *M. catarrhalis* changed significantly, showing increased resistance to ampicillin (Mikucka et al. 2000). As expected, in this study *M. plurianimalium*, susceptibility to ampicillin and penicillin among isolates was extremely low. *M.plurianiamalium* was also ceftiofur resistance. This resistance pattern emphasizes the importance of monitoring *M. pluranimalium* closely and considering alternative therapies when β-lactams are ineffective. Like *M. catarrhalis* (Mikucka et al. 2000), *B. bronchiseptica* can produce beta lactamase enzymes and reduced membrane permeability to ceftiofur (Chander et al., 2011). Dayao et al. (2014) found resistance to ceftiofur in *B. bronchiseptica* in Australia. Nonetheless, the ceftiofur AMR pattern was also detected in *R. nasimurium, A. rossi* and PM. Noteworthy, the presence of strains showing resistance to ceftiofur, placed in category B (EU 2019/6), turns on the warning light to avoid AMR burden in humans (Rhouma et al. 2022). On the other hand, it poses veterinary medicine in a very sensitive situation which could hamper the care of animals and generate severe welfare issues (Vilaró et al. 202).

Considering the resistance patterns found in the community living in the mucosa of pigs in Europe and worldwide, reduction of AMU in pigs is imperative to maintain animal health (Holmer et al. 2019). In addition, for preventing the development of AMB resistance, reduction of the selective pressure boosted by AMB may allow commensals to play a role in animal immunity, particularly some endogenous species in the nasal mucosa of piglets, which may enhance animal immunity throughout life.

Conclusion

Identical patterns found in nosocomial newborn piglet inhabitants suggest the exchange of these strains from the colostrum and mother's breath. However, diverse AMR patterns among isolates from sow vaginal and nasal swabs, piglet nasal swabs and colostrum highlight that bacterial AMB susceptibilities should not be generalized even for bacteria located in the same ecological niche. For this reason, the inclusion of commensals of veterinary microbiota and not only pathogens are important to establish breakpoints (ECOFFs) and fill gaps in antimicrobial susceptibility monitoring programs in the One Health approach.

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CLOSING REMARKS

Restrictive legislation banning AMBs as growth promoters in livestock is on the rise (Lentz, 2022), however, oral medication is still the most common route of AMU in pig production (Lekagul et al. 2019, Bosman et al. 2022). Although, metaphylactic treatments are no longer recommended (EU 2022/1255), its strategy is still used to control the occurrence of *S. suis* (Correa-Fiz et al. 2020) or *G. parasuis* (Costa-Hurtado et al. 2020) or even when it fails any of the preventive measures included in the improvement of environmental conditions or vaccination programs (Vilaró et al. 2023).

In this context, AMU in feed at early stages of life is a practice in porcine production in Spain (Correa-Fiz et al. 2019). Also, prophylactic use for acclimation of sows is still often in Brazil (Dutra et al., 2021). In a matter of fact, AMB is used as part of the infrastructure that sustains health and high levels of production in pig farms (Albernaz-Gonçalves et al. 2022). Indeed, both resistance patterns' studies highlight the need for targeted AMB therapies based on AST (Vilaró et al. 2020; Somogyi et al. 2023).

Most likely, this misuse of AMU contributed to high rates of AMR, particularly to tetracyclines, macrolides, b-lactams, including third generation cephalosporin (Uruén et al. 2024). Ceftiofur, third generation cephalosporin is one of the most used AMB for treating bacterial respiratory infections in swine due to its broad-spectrum activity, effectiveness, and practical application (Smith & Johnson, 2020). In some Canadian pig herds (Bosman et al. 2022) as well in Spain (Blanco-Fuertes et al. 2023) and Brazil (Dutra et al. 2021) there was routine disease prevention use of ceftiofur in suckling pigs. Notable, we found *A. suis, S. aureus* and *S. suis* isolates (experiment in Brazil) as well *R. nasimurium, M. plurianimalium, A. rossi,* and *P. multocida* (experiment in Spain) resistant to ceftiofur. Conversely, Somogyi et al. (2023) reported ceftiofur outstanding efficacies against *A. pleuropneumoniae, P. multocida,* and *S. suis* isolates.

In line with our results in Brazil research, Serpa et al. (2020) reported a great level of resistance to erythromycin, sulfadiazine/trimethoprim, and tetracycline among bacterial respiratory pathogens isolated from pigs in Brazil. Contrarywise, macrolides and β-lactam resistance observed in Barcelona´s experiment, particularly exhibited by *R.nasimurium, S. Suis,* and *M. plurianimalium,* are not in agreement with Vilaró et al. 2023, which reported stability or increase susceptibility to AMB in pig respiratory pathogens, except for tetracycline.

Meanwhile, tetracycline resistance genes maintenance in swine respiratory pathogens in Spain (Gutiérrez-Martin et al. 2006; Vilaró et al. 2020) and around Europe (El Garch et al. 2016) is related

to the period of life of these genes. Collins & Bowring (2023) reported that in treatment to prevent post-weaning diarrhea, *E. coli* resistance to aminoglycosides appeared to be short-lived, whereas resistance to tetracycline persisted for at least three weeks after treatment was stopped. Mutations that reduce the AMB affinity often result in a fitness cost to the organism, particularly in the absence of the AMB agent (Collins & Bowring, 2023). Indeed, there is a significant correlation between resistance and reduced fitness. Relative fitness can be defined as a reduction in growth rate in vitro or in vivo or in terms of colonization, transmissibility, or virulence. But compensatory mutations restore fitness, retaining resistance; therefore, once resistance is established is likely to persist in the population (Hughes, 2014).

Worldwide concern about the use of AMBs in livestock is leading to a new awareness of AMU among stockholders (Rigueira & Perecmanis, 2022). In fact, international legislation is increasingly restricting the use of AMB as metaphylactic uses or those critical for humans (EU 2019/6). Choosing alternatives to AMU and good practices is a wiser course of action (Patience & Ramirez, 2022). Reduction in AMU in veterinary medicine requires the implementation of preventive measures, based on alternative tools such as vaccination and other strategies to guarantee a beneficial microbial colonization of the animals (Costa-Hurtado et al. 2020). On the other hand, AMB treatment, gaseous ammonia concentration, diet and floor type are amongst the recognized environmental factors (Pirolo et al. 2021) which affects the composition of the microbiome flora to an unstable microbiome (Blanco-Fuertes et al. 2023) undermining loss of the beneficial and commensal microbes that prevent the colonization of opportunistic pathogens (Elgamal et al. 2021), thus the animal's immunity is compromised.

In this context, the nasal microbiome´s studies may help to evaluate the impact of the selective pressure caused by AMB and further ahead, a parameter of health status. To access the pig nasal microbiota, the first step is to estimate the prevalence of upper tract respiratory inhabitants. Espinosa-Gongora (2016) reported that nasal microbiome of pigs that are not colonized with *S. aureus* harbors several species/taxa that are significantly less abundant in pig carriers, suggesting that the nasal microbiota may play a role in the individual predisposition to *S. aureus* nasal carriage in pigs. Hopkins et al. (2018) elucidated by community diversity why some pigs with the same set of risk factors from weaning to nursery remained healthy carriers, while others developed clinical *S. suis* disease.

Yet with few limitations, this research was the first epidemiological analysis on sow nasal microbiome in the Federal District, Brazil. One of the limitations could be the rayon-tipped swabs used in the research to collect the nasal samples. Takeuti´s (2017) findings indicate that flocked

nylon swabs have greater material absorption and sensitivity in detecting Mhyo compared to rayon-tipped swabs. Another limitation was that the report use of AMBs was based on survey questionnaires and not on prescription reviews, due, by the sampling period, the veterinary prescription had not been implemented yet. Last, in this study it was not possible to evaluate AMU with lower nasal community diversity.

On the other hand, this unbalanced microbiome associated with AMBs was observed in others research taking in place in IRTA-CReSA. Recent studies are advancing the understanding of this complex route and point to alternatives to AMU to slow AMR, for example, nasal colonizers with potential probiotic properties (Lorenzo et al. 2018; López-Serrano et al. 2020; Obregón-Gutierrez et al. 2024).

Moreover, resistance trends´ study can be accessed through epidemiologic surveillance of AMR (Gutiérrez-Martín et al. 2024). Hence, to increase microbial susceptibility quantitative knowledge, MIC test was performed by broth microdilution during sandwich doctorate. However, granting MIC data for the AMBs without ECOFF is difficult to interpret (El Garch et al. 2016). Even though, in Spain, epidemiological approach has been developed to carry out the prudent use of AMBs in pigs for respiratory pathogens at a large scale in Spain since 2018 (Vilaró et al. 2020).

Accordingly, AMR monitoring programs are important for future evaluation of interventions through AMU more conscious, emphasizing animal welfare, biosecurity, good management practices and alternatives to AMB in pig farms (Rigueira & Perecmanis, 2024). By minimizing environmental and management stressors, pigs can become more immunocompetent and prepared to overcome pathogenic challenges. This outcome can contribute to reducing AMU and the risk of AMR (Albernaz-Gonçalves et al. 2022).

Whereas resistance varied for some pathogens over time in response to usage (Homer et al. 2019), one of the great challenges in this line of research is that reducing both AMU and AMR may not occur at the same time (Hughes, 2014). Indeed, bacitracin, the longest AMB period used reported in Brazilian study, was found the highest resistance even not being used anymore, raising questions if resistance traits can persist over time. Vilaró et al. (2023) justify this with phylogenetic studies showing an epidemiological link between the origin (grandmother's farm) of the isolates and the phylogenetic group supported in the production/breeding pyramid. In a previous report, Guitart-Matas et al. (2022) observed epidemiological evidence suggesting vertical transmission of these resistance traits within integrated systems. That is, even in the absence of AMB, resistant strains

may persist if compensatory mutations arise or if resistance genes are associated with other advantageous traits such as plasmid carriage (Hughes, 2014).

According to all evidence, AMR is a multi-faceted cross-border threat to health that cannot be tackled by one sector independently. Hernandez-Garcia et al. argued that combination of different approaches enhances the information obtained from the isolates associated from disease and non-disease with different resistance profiles. Tackling AMR requires a global level of collaboration, including high compromises between countries (EU 2019/6). AMR patterns can change with time, and it is one of the reasons to monitor it across time (Vilaró et al. 2020). The development and application of practices of AMB stewardship in animal is a critical part of the huge global effort to address AMR (Prescott, 2017). Therefore, future studies to enhance PAN BR AGRO, such including sow nasal swabs in AMR program, may strengthen the Brazilian program in One Health framework.

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APPENDICE

Annex 1 CEUA Certification

Universidade de Brasília
Comissão de Ética no Uso Animal

Brasília, 15 de maio de 2023.

CERTIFICADO

Certificamos que o projeto intitulado **"ISOLAMENTO, IDENTIFICAÇÃO MOLECULAR E PERFIL DE RESISTÊNCIA EM AGENTES BACTERIANOS COLETADOS DA CAVIDADE NASAL DE MATRIZES SUÍNAS NO DISTRITO FEDERAL EM ASSOCIAÇÃO À ANÁLISE DOS FATORES DE VULNERABILIDADE"**, SEI n. 23106.022976/2023-55, sob responsabilidade da pesquisadora Simone Perecmanis, encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal - CONCEA, foi avaliado e aprovado pela Comissão de Ética no Uso Animal (CEUA) da Universidade de Brasília, na 192ª reunião ordinária, em 24/04/2023. Este projeto foi aprovado para utilização de *Sus scrofa domesticus* (50 fêmeas), provenientes de propriedades rurais ou granjas comerciais do Distrito Federal.

O presente certificado é válido pelo período de 15/06/2023 a 15/06/2026.

Universidade de Brasília UnB

Carina Krewer

Dra. Carina da Costa Krewer

Coordenadora da CEUA – UnB

Comissão de Ética no Uso de Animais CEUA

*Este documento se restringe à avaliação ética do projeto supracitado e não substitui outras licenças e permissões que porventura se façam necessárias.

Annex 2 Chapter 2

TABLE S1 - Resistances found in each isolate

Table S1a. Resistances found in each isolate are indicated with a 1 below the antibiotic tested. 0 indicates susceptibility.

Bacterial species	Farm	#ATM used in farm	# isolates	AMC	AMI	AMO	AMP	BC	CFE	CFL	CLI	CTF	DOX	ENO	ERI	FLF	GEN	MBF	NEO	NOR	PEN	SUL	SUT	TET	TLS	TUL
Actinobacillus suis	A	5	1	1	1	1	0	1		1		1	0	1		1	1	1	1		1	0	1	1	1	1
Actinobacillus suis	B	3	1	1	1	1	1	1		1		1	1	1		1	1	1	1		1	1	1	1	1	1
Actinobacillus suis	B	3	1	1	1	1	0	1		0		0	0	1		1	0	0	1		1	0	1	1	1	1
Actinobacillus suis	C	2	1	1	1	1	0	1		1		0	0	1	0	1	1	1	1		1	0	0	0	0	0
Actinobacillus suis	E	1	1	1	1	1	0	1		1		0	0	1	0	1	1	1	1		1	0	1	1	1	1
Actinobacillus suis	G	1	1	1	1	1	1	1		0		1	1	1	1	1	0	0	1		1	1	1	1	1	1
Actinobacillus suis	H	2	1	1	1	1	1	1		0		1	1	1	1	1	0	0	1		1	1	1	1	1	1
Bordetella bronchiseptica	D	2	1	1	0	1	0	1		1		0	0	1	1	0	0	1	0		1	1	1	1	0	0
Bordetella bronchiseptica	E	1	1	0	0	1	0	1		1		0	1	1	1	0	0	1	0		1	1	1	1	1	0
Bordetella bronchiseptica	E	1	1	1	0	1	0	1		1		0	0	1	1	0	0	1	0		1	1	1	1	0	0
Bordetella bronchiseptica	F	1	1	0	0	1	1	1		0		0	0	0	0	1	0	0	0		1	1	1	0	0	1
Bordetella bronchiseptica	G	1	1	0	0	1	0	1		1		0	0	0	1	1	0	1	0		1	1	1	1	1	1
Citrobacter freundii	E	1	1	0	0	1	1	0			1	0	1	0	1	1	0	1	0		1	0	1		1	1
Citrobacter freundii	G	1	1	0	0	1	1	0			1	0	1	0	1	0	0	0	0		1	0	1		1	0
Citrobacter freundii	I	2	1	0	0	1	1	0			1	1	1	1	1	1	0	0	0		1	1	0	1	1	0
Corynebacterium suis	A	5	1	0	1	1	1	1			0	1	1	1	0	0	0	1	1	1	1	1	0	1	1	0
Corynebacterium suis	H	2	1	0	1	1	1	1			0	1	1	1	0	0	0	1	1	1	1	1	0	1	1	0
Escherichia coli	C	2	1	0	0	0	1	1	1	0	1	0	1		1	1	0	0	0		1	0	0	1	1	0
Escherichia coli	E	1	1	0	0	0	1	1	1	0	1	0	1		1	1	1	1	0		1	0	0	0	1	0
Escherichia coli	F	1	1	0	1	0	1	1	1	0	0	0	1		1	1	1	1	0		1	0	0	1	1	1
Klebsiella aglomerans	A	5	1	0	0	1	1	1	1		1	1	1		1	1	1	1	0	1	0	1	0	0	0	
Klebsiella aglomerans	A	5	1	0	0	1	1	1	1		1	1	1		1	1	1	1	0	1	1	1	0	0	0	1
Klebsiella aglomerans	D	2	1	0	0	1	1	1	1		1	0	1		0	0	0	0	0	0	0	0	1	1	1	0
Klebsiella aglomerans	D	2	1	0	0	1	1	1	1		1	0	1		0	1	1	0	0	0	0	1	1	1	1	1
Klebsiella aglomerans	E	1	1	0	0	1	1	1	1		1	0	1		1	1	0	0	0	0	0	0	0	0	0	0
Klebsiella aglomerans	E	1	1	0	0	1	1	1	1		1	0	1		1	1	1	1	0	1	0	1	1	1	1	0
Klebsiella aglomerans	F	1	1	0	0	1	1	1	1		1	0	1		1	1	1	1	0	1	0	0	0	0	0	0
Klebsiella aglomerans	F	1	1	0	0	1	1	1	1		1	1	1		1	1	1	1	0	1	0	1	1	1	1	0
Klebsiella aglomerans	G	1	1	0	0	1	1	1	1		1	0	0		1	1	0	0	1	1	1	1	1	0	1	0
Klebsiella aglomerans	J	3	1	1	0	1	1	1	1		1	0	1		1	1	0	1	0	1	1	1	1	0	1	
Klebsiella oxitoca	J	3	1	0	0	1	1	1	1	0	0	1	1	1	1	0	0	0	0		1	1	0	1	0	0
Klebsiella oxitoca	J	3	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	0	1		1	1	1	1	1	0
Klebsiella pneumoniae	B	3	1	1	0	1	1	1	1	0	1	0	1	1	1	1	0	0	0		1	1	0	1	1	0
Klebsiella pneumoniae	B	3	1	1	0	1	1	1	1	1	1	1	1	1	1	1	0	1	0		1	1	1	1	1	0
Klebsiella pneumoniae	D	2	1	1	0	1	1	1	1	1	1	0	1	1	1	1	0	0	0		1	1	0	1	1	0
Klebsiella pneumoniae	E	1	1	0	0	1	1	1	0	0	1	0	1	1	1	1	0	0	0		1	1	0	1	1	0
Klebsiella pneumoniae	E	1	1	1	0	1	1	1	1	0	1	1	1	1	1	1	0	1	0		1	1	1	1	1	0
Klebsiella pneumoniae	E	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1		1	1	1	1	1	0
Klebsiella pneumoniae	F	1	1	0	1	1	0	1	1	1	1	0	1	1	1	1	0	0	1		1	1	0	1	1	0
Klebsiella pneumoniae	G	1	1	0	0	0	0	1	1	1	1	0	0	0	0	1	0	0	0		1	0	0	1	1	0
Klebsiella pneumoniae	G	1	1	0	0	1	1	1	1	1	1	0	0	0	1	1	0	0	0		1	0	1	1	1	0
Klebsiella pneumoniae	G	1	1	0	0	1	1	1	1	1	1	0	1	0	1	1	0	0	1		1	1	1	1	1	1
Klebsiella pneumoniae	G	1	1	0	1	1	1	1	1	1	1	0	1	0	1	1	0	0	1		1	1	1	1	1	1
Klebsiella pneumoniae	H	2	1	0	0	1	0	1	0	0	1	0	0	0	1	0	0	0	0		1	0	0	1	1	0
Klebsiella pneumoniae	H	2	1	1	0	1	1	1	0	0	1	0	1	1	1	1	0	0	0		1	1	1	1	1	0
Klebsiella pneumoniae	I	2	1	0	0	1	1	1	0	0	1	0	1	1	0	1	0	0	0		1	1	0	1	1	0
Klebsiella pneumoniae	I	2	1	1	0	1	1	1	1	0	1	1	1	1	1	1	0	0	0		1	1	0	1	1	0
Klebsiella pneumoniae	I	2	1	1	0	1	1	1	1	0	1	1	1	1	1	1	0	1	0		1	1	1	1	1	0

Empty cells: not done

AMC: Amoxicilina/Ácido clavulánico; AMI: Amikacina; AMO: Amoxicillin; AMP: Ampicillin; BC: Bacitracin; CFE: Cephalexin; CFL: Cephalothin; CLI: Clindamycin; CFT: Ceftiofur; DOX: Doxycycline; ENO: Enrofloxacin; ERI: Erythromycin; FLF: Florfenicol; GEN: Gentamicin; MBO: Marbofloxacin; NEO: Neomycin; NOR: Norfloxacin; PEN: Penicillin; SUL: sulfametoxazol; SUT: sulfametoxazol-trimetoprim; TET: Tetracycline; TUL: Tulathromycin; TLS: Tylosin.

.....Cont.....

ANNEX 2 CHAPTER 2

TABLE S1 - Resistances found in each isolate

Table S1b. Resistances found in each isolate are indicated with a 1 below the antibiotic tested. 0 indicates susceptibility.

Bacterial species	Farm	#ATM used in farm	# isolates	AMC	AMI	AMO	AMP	BC	CFE	CFL	CLI	CTF	DOX	ENO	ERI	FLF	GEN	MBF	NEO	NOR	PEN	SUL	SUT	TET	TLS	TUL
Micrococcus luteus	C	2	1	0	0	1	1	1	0	0	0	0	0	1	0	1	0	0	0			1	0	0	1	1
Micrococcus luteus	D	2	1	0	0	1	1	1	0	0	1	0	0	1	0	1	1	0	0			1	1	1	1	1
Micrococcus luteus	F	1	1	0	0	0	0	1	1	0	1	0	1	1	0	1	0	0	0			1	0	0	0	0
Micrococcus luteus	G	1	1	0	0	0	1	1	1	0	1	0	1	0	0	1	0	0	0			1	0	1	1	1
Micrococcus luteus	I	2	1	0	0	0	0	1	0	0	0	0	0	0	0	1	1	0	0			1	0	0	1	0
Micrococcus luteus	I	2	1	0	0	0	0	1	0	0	1	0	0	0	0	1	1	0	0			1	0	0	1	0
Micrococcus luteus	I	2	1	0	0	0	0	1	0	0	1	1	0	0	1	1	1	0	0			1	1	0	1	1
Pasteurella multocida	A	5	1	0	0	0	0	1	1	1	0	0	0	0	0	0	0	1	0		1	0	0	1	1	0
Pasteurella multocida	A	5	1	1	0	0	0	1	1	1	1	0	1	1	1	1	1	1	1		1	0	1	1	1	1
Pasteurella multocida	B	3	1	1	0	1	1	1	1	1	1	0	1	1	1	1	0	1	0		1	0	1	1	1	1
Pasteurella multocida	E	1	1	1	0	1	0	1	1	1	1	0	1	1	1	0	1	1	0		1	1	1	1	1	0
Pasteurella multocida	G	1	1	1	0	0	0	1	1	1	1	0	1	1	1	1	1	1	1		1	0	0	1	1	1
Pasteurella multocida	H	2	1	0	0	0	0	1	1	1	0	0	0	0	0	0	0	1	0		1	0	1	1	1	0
Proteus vulgaris	A	5	1	0	0	0	0	1	1	1	1	0	1	0	0	0	0	0	0	1	0	1	0	1	1	0
Proteus vulgaris	B	3	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0				
Proteus vulgaris	B	3	1	0	0	0	1	1	1	0	0	0	1	1	1	0	0	1	0	1	1	1				
Proteus vulgaris	D	2	1	0	0	1	1	1	1	1	1	1	1	1	1	0	0	0	0	1	1	0	1	1	0	0
Proteus vulgaris	F	1	1	0	0	0	1	1	1	0	0	0	1	1	1	0	0	1	0	1	1	1	1	1	1	1
Proteus vulgaris	F	1	1	0	0	1	1	1	1	0	0	0	1	1	1	0	0	1	0	1	1	1	1	1	1	1
Proteus vulgaris	G	1	1	0	0	0	0	1	1	1	1	0	1	0	0	0	0	0	0	1	0	1	0	1	1	0
Proteus vulgaris	H	2	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Proteus vulgaris	H	2	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1	0	1	1	1	1	1	1	1	1
Pseudomonas aeruginosa	G	1	1	1	0	1	1			1	1	0	1		1	1	0	1	1		1	1	1		1	1
Rhodococcus equi	B	3	1	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0
Rhodococcus equi	B	3	1	0	0	0	0	1	0	0	1	1	1	0	0	1	0	0	0	1	1	0	0	1	1	0
Rhodococcus equi	B	3	1	0	0	1	0	1	1	1	1	1	1	1	0	1	1	1	0	1	1	1	1	1	1	0
Rhodococcus equi	C	2	1	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	1	0	0
Rhodococcus equi	C	2	1	0	0	1	0	1	0	1	0	1	1	1	1	1	1	1	1	0	1	1	0	1	0	0
Rhodococcus equi	C	2	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Rhodococcus equi	F	1	1	0	0	0	0	1	1	1	1	1	1	1	0	1	0	1	0	1	1	0	0	1	0	1
Rhodococcus equi	G	1	1	0	0	0	0	1	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Rhodococcus equi	G	1	1	0	0	0	0	1	0	0	1	0	1	0	1	1	0	1	0	0	1	1	1	1	0	0
Rhodococcus equi	H	2	1	0	0	0	0	1	0	0	0	0	1	1	0	1	1	0	0	1	1	0	0	1	1	0
***Salmonella* Typhimurium**	I	2	1	1	0	1	1	1	1	1	1		1	1	1	1	0	0	0	1	1	1	1	1	1	0
***Salmonella* Typhimurium**	J	3	1	1	0	1	1	1	1	1	0		1	0	1	1	0	0	1	1	1	1	1	1	1	0
Staphylococcus aureus	A	5	1	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Staphylococcus aureus	E	1	1	0	0	1	1	1	1	1	1	0	1	0	1	0	0	0	1	1	1	0	0	1	0	1
Staphylococcus aureus	E	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	1	1	1	1	1	1	1	1
Staphylococcus aureus	H	2	1	1	1	1	1	1	0	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1	1	1
Staphylococcus aureus	J	3	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Staphylococcus aureus	J	3	1	0	0	1	1	1	1	1	1	0	1	1	1	1	0	1	1	1	1	0	0	1	1	1
Staphylococcus hyicus	J	3	1	0	0	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	0	1	0	0
Staphylococcus hyicus	J	3	1	0	0	1	1	1	1	0	1	0	0	1	0	1	1	1	0	0	1	1	1	1	1	1

Empty cells: not done

AMC: Amoxicilina/Ácido clavulánico; AMI: Amikacina; AMO: Amoxicillin; AMP: Ampicillin; BC: Bacitracin; CFE: Cephalexin; CFL: Cephalothin; CLI: Clindamycin; CFT: Ceftiofur; DOX: Doxycycline; ENO: Enrofloxacin; ERI: Erythromycin; FLF: Florfenicol; GEN: Gentamicin; MBO: Marbofloxacin; NEO: Neomycin; NOR: Norfloxacin; PEN: Penicillin; SUL: sulfametoxazol; SUT: sulfametoxazol-trimetoprim; TET: Tetracycline; TUL: Tulathromycin; TLS: Tylosin.

.....Cont.....

ANNEX 2 CHAPTER 2

TABLE S1 - Resistances found in each isolate

Table S1C. Resistances found in each isolate are indicated with a 1 below the antibiotic tested. 0 indicates susceptibility.

Bacterial species	Farm	#AMB used in farm	isolate	AMC	AMI	AMO	AMP	BC	CFE	CFL	CLI	CTF	DOX	ENO	ERI	FLF	GEN	MBF	NEO	NOR	PEN	SUL	SUT	TET	TLS	TUL
Staphylococcus coagulase negative	A	5	1	1	0	1	1	1	0	1	1	1	1	0	0	1	1	1	1	1	1	1	1	1	0	0
Staphylococcus coagulase negative	B	3	1	0	0	0	0	1	0	1	0	1	1	1	1	0	1	0	0	0	1	1	1	0	1	0
Staphylococcus coagulase negative	C	2	1	0	0	1	0	1	0	0	0	1	1	0	0	0	1	0	0	0	0	1	0	0	0	0
Staphylococcus coagulase negative	C	2	1	1	0	1	1	1	0	0	0	1	1	0	0	0	1	1	1	0	0	1	1	1	1	0
Staphylococcus coagulase negative	D	2	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Staphylococcus coagulase negative	D	2	1	0	0	1	1	0	0	0	1	1	0	0	0	0	1	0	0	0	0	1	0	0	0	0
Staphylococcus coagulase negative	E	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Staphylococcus coagulase negative	E	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Staphylococcus coagulase negative	E	1	1	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Staphylococcus coagulase negative	E	1	1	0	0	1	1	1	0	0	1	1	0	0	0	1	0	1	0	0	0	0	0	1	1	1
Staphylococcus coagulase negative	F	1	1	0	0	0	1	1	0	0	0	0	0	0	1	0	0	0	1	0	1	1	1	0	1	0
Staphylococcus coagulase negative	F	1	1	0	1	0	1	1	0	0	0	0	1	1	1	0	1	0	1	0	1	1	1	0	1	0
Staphylococcus coagulase negative	G	1	1	0	0	0	0	1	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	1	0
Staphylococcus coagulase negative	G	1	1	0	0	0	1	1	0	0	0	1	1	0	1	0	1	0	0	0	0	1	0	0	1	0
Staphylococcus coagulase negative	G	1	1	0	0	1	1	1	0	0	0	1	1	1	1	0	1	0	0	0	0	1	1	0	1	0
Staphylococcus coagulase negative	G	1	1	0	0	1	1	1	0	1	1	1	1	1	1	0	1	0	0	0	1	1	1	1	1	0
Staphylococcus coagulase negative	G	1	1	0	1	1	1	1	0	1	1	1	1	1	1	1	1	0	0	1	1	1	1	1	1	1
Staphylococcus coagulase negative	H	2	1	0	0	0	0	1	0	0	0	1	1	1	0	1	1	0	0	0	1	1	0	0	1	0
Staphylococcus coagulase negative	H	2	1	0	0	0	0	1	0	0	0	1	1	1	1	1	1	0	0	0	1	1	0	0	1	0
Staphylococcus coagulase negative	H	2	1	0	0	1	0	1	0	0	1	1	1	1	1	1	1	0	0	0	1	1	1	1	1	1
Staphylococcus coagulase negative	I	2	1	0	0	0	0	1	0	0	0	1	1	1	1	0	1	0	0	0	0	1	1	0	1	0
Staphylococcus coagulase negative	I	2	1	0	1	1	1	1	0	1	0	1	1	1	1	0	1	0	0	0	1	1	1	0	1	1
Staphylococcus coagulase negative	J	3	2																							
Staphylococcus coagulase positive	A	5	1	0	0	1	0	1	1	1	1	1	1	0	0	1	0	0	0	1	1	1	1	1	0	1
Staphylococcus coagulase positive	B	3	1	0	0	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	0	0	1	1	0
Staphylococcus coagulase positive	C	2	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1
Staphylococcus coagulase positive	E	1	1	0	0	1	0	1	1	1	1	1	1	1	0	1	0	0	0	1	1	0	0	1	1	0
Staphylococcus coagulase positive	E	1	1	0	0	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	0	1	1	0
Staphylococcus coagulase positive	F	1	1	0	0	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	0	0	1	0	0
Streptococcus suis	A	5	1	0	0	0	0	1	1	1	1	1	1	1	0	1	0	1		1	1	1	0	1	0	0
Streptococcus suis	B	3	1	1	0	1	0	1	1	1	1	1	1	1	0	1	1	0		1	1	0	0	1	0	0
Streptococcus suis	C	2	1	1	1	0	1	1	1	1	1	1	1	1	0	1	0	0		1	1	1	0	1	0	1
Streptococcus suis	C	2	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0	0		1	1	1	1	1	1	1
Streptococcus suis	G	1	1	0	0	0	0	1	1	1	1	1	1	1	0	1	0	1		1	1	1	0	1	0	0
Yersinia enterocolitica	E	1	1	0	0	1	0	1		0	0	0	0	1	1	1	0	0	0	0	1	0	0	0	1	0
Yersinia enterocolitica	E	1	1	0	0	1	1	1		0	1	0	1	1	1	1	0	0	0	0	1	1	1	1	1	0
Yersinia enterocolitica	E	1	1	1	1	1	1	1		0	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1
Yersinia enterocolitica	G	1	1	0	0	0	0	1		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Yersinia enterocolitica	G	1	1	0	0	0	0	1		0	0	0	0	0	0	1	0	0	0	0	1	0	0	1	0	0
Yersinia enterocolitica	G	1	1	0	0	0	0	1		0	1	0	0	0	0	1	0	0	0	0	1	0	1	1	1	0
Yersinia enterocolitica	G	1	1	0	1	1	0	1		0	1	0	1	0	1	1	0	1	0	1	1	1	1	1	1	1
Yersinia enterocolitica	H	2	1	1	0	1	1	1		1	1	0	1	1	1	1	1	1	1	1	1	1	1	0	1	0
		# resistencias		36	22	86	77	121	64	57	84	54	96	74	74	91	52	57	36	46	99	85	67	90	94	43
		# test		132	132	132	132	131	106	117	120	130	132	118	129	132	132	132	127	82	125	132	130	127	130	128
		%		**27,3**	**16,7**	**65,15**	**58,3**	**92,4**	**60,4**	**48,7**	**70**	**41,5**	**72,7**	**62,7**	**57,4**	**68,9**	**39,4**	**43,2**	**28,3**	**56,1**	**79,2**	**64,4**	**51,5**	**70,9**	**72,3**	**33,6**

Empty cells: not done

AMC: Amoxicilina/Ácido clavulánico; AMI: Amikacina; AMO: Amoxicillin; AMP: Ampicillin; BC: Bacitracin; CFE: Cephalexin; CFL: Cephalothin; CLI: Clindamycin; CFT: Ceftiofur; DOX: Doxycycline; ENO: Enrofloxacin; ERI: Erythromycin; FLF: Florfenicol; GEN: Gentamicin; MBO: Marbofloxacin; NEO: Neomycin; NOR: Norfloxacin; PEN: Penicillin; SUL: sulfametoxazol; SUT: sulfametoxazol-trimetoprim; TET: Tetracycline; TUL: Tulathromycin; TLS: Tylosin.

Annex 3 Chapter 2
Table S2 - Period use of antimicrobial agent in sow´s farm and percentual of resistance

Table S2 - Antimicrobial agent (atm); Period use in sow´s farm (years); Results of resistance: (R), n° (%)

Atm	Years	R (%)
Bacitracin	9	124 (93.9)
Florfenicol	7	101 (76.5)
Clindamycin	7	98 (74.2)
Penicillin	7	95 (72.0)
Tetracycline	7	94 (71.2)
Amoxicillin	5	93 (70.5)
Enrofloxacin	5	93 (70.5)
Tylosin	5	91 (68.9)
Norfloxacin	4	90 (68.2)
Doxycycline	4	89 (67.4)
Ampicillin	4	88 (66.7)
Cefalexin	4	82 (62.1)
Tulatromycin	3	79 (59.8)
Cefalotin	3	77 (58.3)
Erythromycin	3	76 (57.6)
Ceftiofur	3	75 (56.8)
Sulfa	3	74 (56.1)
Neomycin	2	63 (47.7)
Sulfa + trimethoprim	2	59 (44.7)
Marbofloxacin	1	47 (35.6)
Gentamicin	1	37 (28.0)
Amoxicilin+Ac. Clavulanic	1	24 (18.2)
Amikacin	1	24 (18.2)
TOTAL	9	132(100)

Annex 4 Chapter 2

TABLE S3. Resistance per species or per each farm

Table S3. Number of isolates from each bacterial species obtained in each farm and the associated percentage of resistance, calculated as the number of tests giving a resistance result with respect to the total number of tests in each bacterial species (resistance per species) or each farm (resistances per farm).

Bacterial species	Farm A	Farm B	Fram C	Farm D	Fram E	Farm F	Fram G	Farm H	Fram I	Farm J	resistence per species	
Actinobacillus suis	1	2	1		1		1	1			**78**	1
Bordetella bronchiseptica				1	2	1	1				54	2
Citrobacter freundii					1		1		1		53	3
Corynebacterium suis	1							1			67	4
Escherichia coli			1		1	1					54	5
Pantoea agglomerans	2			2	2	2	1			1	62	
Klebsiella oxytoca										2	68	
Klebsiella pneumoniae		2		1	3	1	4	2	3		65	
Micrococcus luteus			1	1		1	1		3		39	
Pasteurella multocida	2	1			1		1	1			61	
Proteus vulgaris	1	2		1		2	1	2			46	
Pseudomonas aeruginosa							1				**83**	
Rhodococcus equi		3	3			1	2	1			45	
Salmonella **Typhimurium**									1	1	**75**	
Staphylococcus aureus	1				2			1		2	66	
Staphylococcus hyicus										2	70	
Staphylococcus coagulase n	1	1	2	2	4	2	5	3	2		40	
Staphylococcus coagulase p	1	1	1		2	1					**71**	
Streptococcus suis	1	1	2				1				68	
Yersinia eneterocolitica					3		4	1			49	
ırm antimicrobial resistance	64,8	57,7	58,2	47,1	55,2	56,1	50,4	56,1	53,9	61,8		

Annex 5 Chapter 3

Chapter III Work table - Not diluted ≤ 50 ng/µl: 5N3P *R. nasimurium*; 9N2S *M. plurianimalium;* 8V1S *S. suis*

Table S1 - Origin type, amount DNA and TRIS dilution.

Cod	Bacterial specie	Sample	*DNA (µl)*	*Tris (µl)*
2N1S	*R. nasimurium*	Sow nasal	2,4	47,6
7N1S	*R. nasimurium*	Sow nasal	4,6	45,4
3N2S	*R. nasimurium*	Sow nasal	0,9	49,1
1C1S	*R. nasimurium*	Colostrum	8,9	41,1
1C3S	*R. nasimurium*	Colostrum	6,2	43,8
8C4S	*R. nasimurium*	Colostrum	4,9	45,1
6C1S	*R. nasimurium*	Colostrum	23,2	26,8
10C1S	*R. nasimurium*	Colostrum	2,6	47,4
1N1P	*R. nasimurium*	Piglet nasal	28,2	21,8
4N1P	*R. nasimurium*	Piglet nasal	2,1	47,9
1N2P	*R. nasimurium*	Piglet nasal	12,7	37,3
5N1P	*R. nasimurium*	Piglet nasal	11,4	38,6
10N1P	*R. nasimurium*	Piglet nasal	11,7	38,3
7N2P	*R. nasimurium*	Piglet nasal	6,5	43,5
13N1P	*R. nasimurium*	Piglet nasal	8,9	41,1
15N1P	*R. nasimurium*	Piglet nasal	5,9	44,1
16N1P	*R. nasimurium*	Piglet nasal	3,8	46,2
15N2P	*R. nasimurium*	Piglet nasal	4,5	45,5
17N2P	*R. nasimurium*	Piglet nasal	28,6	21,4
20N1P	*R. nasimurium*	Piglet nasal	2,3	47,7
24N1P	*R. nasimurium*	Piglet nasal	5,5	44,5
21N2P	*R. nasimurium*	Piglet nasal	4,7	45,3
28N2P	*R. nasimurium*	Piglet nasal	18,0	32,0
11N2S	*M. plurianimalium*	Sow nasal	12,7	37,3
13N2S	*M. plurianimalium*	Sow nasal	1,6	48,4
8C2S	*M. plurianimalium*	Colostrum	2,6	47,4
8C5S	*M. plurianimalium*	Colostrum	2,2	47,8
1N3S	*M. plurianimalium*	Sow nasal	1,6	48,4
5N3S	*M. plurianimalium*	Sow nasal	1,0	49,0
8N2S	*M. plurianimalium*	Sow nasal	2,5	47,5
11C3S	*M. plurianimalium*	Sow nasal	8,7	41,3
1V3S	*A.rossi*	Sow nasal	3,2	46,8
3V2S	*A.rossi*	Sow nasal	11,3	38,7
7V1S	*A.rossi*	Sow nasal	2,0	48,0
8V2S	*A.rossi*	Sow nasal	3,2	46,8
12V3S	*A.rossi*	Sow nasal	1,5	48,5
15N3P	*S. dysgalactiae*	Piglet nasal	6,9	43,1
9V1S	*S. dysgalactiae*	Vaginal	2,8	47,2
3V1S	*S. dysgalactiae*	Vaginal	1,9	48,1
5N1S	*S. dysgalactiae*	Sow nasal	17,4	32,6
14V2S	*S. suis*	Vaginal	10,4	39,6
12N2S	*S. suis*	Sow nasal	2,5	47,5
12N3S	*P. multocida*	Sow nasal	7,9	42,1
13N3S	*P. multocida*	Sow nasal	40,6	9,4
7V2S	*S. aureus*	Sow nasal	5,7	44,3
15N4P	*S. aureus*	Piglet nasal	2,7	47,3
2V2S	*S. aureus*	Vaginal	1,7	48,3
14N3S	*S. aureus*	Sow nasal	4,4	45,6
3N1S	*S. aureus*	Sow nasal	5,3	44,7
23N1P	*S. haemoliticus*	Sow nasal	3,3	46,7
13C1S	*S. haemoliticus*	Colostrum	15,9	34,1
9N1P	*S. pseudoepidermidis*	Piglet nasal	6,4	43,6
7V3S	*S. pseudoepidermidis*	Vaginal	28,2	21,8

Annex 6 Chapter 3
Instructions for Use SensititreTM

013-VET-CID10525
Revision Date: Jan , 2020

For Veterinary Use

18-24 hour MIC and Breakpoint Susceptibility Plates.
Thermo ScientificTM SensititreTM 18-24 hour MIC and Breakpoint Susceptibility Plates

For full plate information, including plate layout, QC information, Interpretative criteria, performance data and references please refer to www.trekds.com/techinfo. The plate code and batch number will be required.

INTENDED USE

The Sensititre susceptibility system is a microversion of the classic broth dilution method and can provide qualitative (Susceptible or Resistant) and quantitative Minimum Inhibitory Concentration (MIC) results in a dried plate format. TREK Diagnostic Systems manufactured broth has only been validated with Sensititre Products.

SUMMARY AND PRINCIPLES OF USE

Each plate is dosed with antimicrobial agents at appropriate dilutions. Results can be read manually by visual reading of growth or automatically on an ARIS OptiRead using fluorescence The technology involves the quantitative detection of bacterial growth by monitoring the activity of specific surface enzymes produced by organisms. The fluorescence substrates are either dried in the plate. Only plates which have the format name suffixed with F can be read automatically.

PRECAUTIONS

Only instruments supported by Sensititre (i.e. a simple manual viewer, Vizion, OptiRead and ARIS) must be used to report results with CE IVD and FDA cleared Sensititre products. Any other system used will not be supported. Results should be used as an aid in selecting the drug of choice for treatment. This product is for in vitro diagnostic use and should be used by properly trained personnel. Precautions should be taken against the dangers of microbiological hazards by properly sterilizing specimens, containers, media, and test plates after use. Directions should be read and followed carefully.

STORAGE AND SHELF LIFE

The plates should be stored at room temperature (15-25°C) away from direct sunlight and direct heat. Each plate is individually packaged in foil and a silica gel desiccant. Do not use the plate if past its expiration date, the desiccant color is not orange or the foil pouch is damaged. Inoculate plate within 5 hours of removal from pouch.

PROCEDURE

Materials included
 Sensititre™ plate with substrate or without substrate in wells.
 Adhesive seal

Materials not included [TREK Inc Product Code]:
 Sensititre™ Demineralized water [T3339]
 5 ml and 11ml Sensititre ™cation adjusted Mueller-Hinton broth with TES (CAMHBT) [T3462]

 11ml Sensititre™ cation adjusted Mueller-Hinton broth with lysed Horse blood (CAMHBT+LHB) [CP112-10]
 11ml Sensititre™ Veterinary Fastidious Medium (VFM) [T3460]
 11ml Sensititre™ Mueller-Hinton Fastidious Medium with Yeast Extract (MHF-Y) [T3461]
 Doseheads (for use with Sensititre™ AIM) [E3010]
 Sensititre™ AIM[V3020]
 Sensititre™ ARIS™ [V3090] / OptiRead™ [V3030]
 Sensititre™ Vizion™ [V2021]
 Sensititre™ Nephelometer [V3011]
 Manual viewer [V4007]
 0.5 McFarland polymer turbidity standard [E1041]
 1 µl and 10µl calibrated loops
 50µl and 100µl pipettor and disposable tips
 Quality control organisms
 Incubator 34-36°C, non CO2
 Vortex mixer
 CO2 generator pack or CO2 incubator
 Agar plates
 Current CLSI, EUCAST, or local guideline documents

SELECTION OF SUSCEPTIBILITY TEST BROTH
Use Sensititre approved CAMHBT for non-fastidious Gram-negative and Gram-positive isolates.
Use Sensititre approved CAMHB+LHB for reading of *S. pneumoniae* isolates.

Only use broth pre-qualified for automated reading of *S. pneumoniae*
Use only Sensititre approved VFM or MHF-Y for *H. somni* (formerly *H. somnus*) and *A. pleuropneumoniae* isolates

Note: Please refer to CLSI VET01 and VET08 or additional details on how to inoculate and read *Histophilus somni* and *Actinobacillus pleuropneumoniae* isolates.

Sensititre broths are performance tested for use in Sensititre susceptibility products.
Use only Sensititre broth for testing Tulathromycin.

INOCULATION PROCEDURES.
Allow all broths to come up to room temperature before use.
Check the carton label for the reconstitution volume of the well.

Preparation of Inoculum for Sensititre Plates

1. For all plates that are to be read manually or plates with substrates that are to be read on the ARIS / OptiRead, pick 3-5 colonies from the primary agar plate and emulsify in demineralized water (or Mueller Hinton broth for *H. somni* and *A. pleuropneumoniae*) and adjust to a 0.5 McFarland standard. Mix well. Colonies of *H. somni* and *A. pleuropneumoniae* should be taken from an overnight (20 to 24 hours) chocolate agar plate incubated in a CO2 incubator.

a. For all non-fastidious aerobic organisms with the exception of Proteus spp.,
Using a calibrated pipette transfer 10µl of the suspension into a tube of 11mL Sensititre Mueller-Hinton broth to give an inoculum of 1x105 cfu/mL **OR** transfer 30 µl of the suspension into an

11 ml tube of cation adjusted Mueller-Hinton broth with TES buffer to ensure detection of heteroresistant isolates among Staphylococcus spp. and Enterococcus spp.
For an inoculum of 5x105 cfu/mL., transfer 50µl of the suspension into a tube of 11mL Sensititre Mueller-Hinton broth.

b. For Proteus spp.
Using a calibrated pipette transfer 1µl of the suspension into a tube of 11mL Sensititre Mueller-Hinton broth.

c. For fastidious organisms
i. For S. pneumoniae isolates transfer 100µl into a tube of 11mL CAMHBT+LHB.
ii. For H. somni and A. pleuropneumoniae isolates, transfer 50µl into a tube of VFM or MHF-Y
iii. Opinions vary as to the best broth for testing H.parasuis. Reference CLSI VET 06 (Methods for Antimicrobial Susceptibility Testing of Infrequently Isolated or Fastidious Bacteria Isolated From Animals) for further information and references for H. parasuis.

d. Increased inoculum method
Depending on the strains tested, an increase to 30 µl may aid in detecting resistance mechanisms. For both Gram positive and negative isolates, the transfer of 30µl of the suspension into an 11 ml Sensititre Mueller-Hinton broth tube results in colony counts which fall within the cleared Sensititre range of 5.0 x 104 and 5.0 x 105 cfu/ml.

3. Vortex or invert the tube 8-10 times.

4. Inoculating and incubating a Sensititre plate
Check the carton label for the reconstitution volume of the well. **A plate intended for 50µl may be dosed with 100µl but the resulting dilutions will be one doubling dilution lower.**

5. Transfer the appropriate volume as stated on the carton label, usually 50µl (100µl for S. pneumoniae) of the broth suspension into each well by either:

5a. **AIM**. Replace the tube cap with a Sensititre single-use dosehead and inoculate the plate according to the AIM instructions.
Remove the test tube/dosehead combination from the AIM within 30 seconds of dosing a plate and store inverted in a rack or discard.

5b. **Manual pipette.** Pour the broth into a sterile seed trough and inoculate the plate using an appropriate pipette (e.g. 8-channel multi-pipettor)

6. Cover the plate with the adhesive seal provided, ensuring that all wells are covered and sealed. Avoid creases as these can lead to skips. Plates intended for CO2 incubation should be covered with a perforated seal

7. Incubate at 34-36°C in a non CO2 incubator for 18-24 hours (20-24 hours for *S. pneumoniae*) ensuring that the plates are stacked no more than three high. In order to ensure detection of vancomycin-resistant *Enterococci* and oxacillin-resistant *Staphylococci,* incubate for 24 hours
For *H. somni* and *A. pleuropneumonia* incubate at 34-36°C in a CO2 incubator or in a gas jar with a CO2 gas pack for 20-24 hours Plates placed in an incubator may require placing in a plastic container with a moistened towel to minimise evaporation.

It is recommended that a periodic check of the control well is done by performing a colony count (Appendix 1). Non-fastidious isolates should have an inoculum of 1x105 cfu/ml (range 5x104 – 5x105) *Proteus* isolates 1x104 cfu/ml (range 5x103 – 5x104) and *S. pneumoniae, H. somni and A. pleuropneumoniae* 5x105 cfu/ml (range 2x105 – 7x105). This check is especially important for *H. somni*, *A. pleuropneumoniae* and *S. pneumoniae* inocula as it can vary depending on the conditions of incubation of the overnight agar plate culture.

Note * Plates intended for 50µL reconstitution can be inoculated with 100 µL **but can only be manually read. Drugs concentrations will be reduced.**

READING TEST RESULTS

1. Automatically

Sensititre plates may be read automatically on the ARIS / OptiRead at 18-24 hours according to the instructions in Sensititre Software Manual with the exception of *H. somni* and *A. pleuropneumoniae* which must be read manually.

Manually

After incubation, results can be read using the Sensititre manual viewer, or the Vizion (see Vizion User Manual). It is not necessary to remove the adhesive seal. Growth appears as turbidity or as a deposit of cells at the bottom of a well. The MIC is recorded as the lowest concentration of antimicrobic that inhibits visible growth. Reading faint growth on Vizion can be improved by use of bright indirect lighting against a dark background. Users can adjust lighting on the Vizion to optimize reading.

The growth control wells should be read first. If any of the control wells do not exhibit growth, the results are invalid. In order to ensure detection of vancomycin-resistant *Enterococci* and oxacillin-resistant *Staphylococci*, results should be interpreted only after a full 24 hours incubation (9, 26). The following points should be noted:

Fading End Points: Most organism/ antimicrobic combinations give distinct end points but with some combinations there may be a gradual fading of growth over 2 to 3 wells. Nevertheless, the end points should be taken as the first well that inhibits visible growth except when reading the results of sulphonamides and Linezolid. In this case, the MIC must be read as an 80-90% decrease in growth compared with the growth of the microorganism in the control well.

Contamination: Contamination may result in a single button of growth in a well with wells on either side showing no growth. Such a single well contamination can be ignored, but if multiple well contamination is suspected, the test should be repeated.

Skips: Occasionally a “skip” may be seen - a well showing no growth bordered by wells showing growth. There are variety of explanations, including contamination, mutation (5) and misaligned dosing. A single skip can be ignored. However, in order to ensure effective antimicrobic therapy NEVER read the skip well as the MIC; always read the lowest well concentration above which there is consistently no growth.

Mixed Cultures: Except as referred to in (a) above, if two end points are seen as a distinct “button” of cells followed by several wells of diffuse growth with the “button” no longer visible (or seen as smaller buttons), there may be a mixed bacterial population. Purity can be checked by sub-culturing the growth onto suitable agar medium. Test results are invalid if a mixed culture is detected.

INTERPRETATION OF RESULTS

MIC Results: The MIC is recorded as the lowest concentration of antimicrobic that inhibits visible growth. If no growth occurs in any well, other than the positive control well, the MIC should be recorded as less than or equal to the lowest concentration of antimicrobic on the plate. The MIC Interpretive Standard Tables in CLSI M7 (26) and M100 (27) and in the CLSI VET01 and VET08 may be used to assign an interpretive category to the MIC results.

MIC Results for ESBL Confirmation: To confirm ESBLs a >3 twofold concentration decrease in an MIC for either the antimicrobial agent tested in combination with clavulanic acid versus its MIC when tested alone = ESBL.

Example: ceftazidime MIC = 8µg/ml ceftazidime/clavulanic acid MIC = 1 µg/ml. For more details refer to CLSI M7 Table 2A.

Breakpoint Results: Breakpoint testing is a broth dilution method for qualitative susceptibility testing. The Sensititre breakpoint system has been developed to provide a simple standardized method for sensitivity testing based on the concept of breakpoint concentrations. A breakpoint is defined as the concentration of an antibiotic that inhibits the growth of sensitive, but not resistant, organisms. The breakpoint concentrations for those antimicrobics on the Sensititre plates are based on the CLSI M100 (27) and VET 08 where available. For most antimicrobics, two concentrations are used, a lower concentration, which represents the upper limit of the susceptible category; and a higher concentration, which represents the upper limit of the intermediate category.
Breakpoint interpretations for antimicrobics present in 1 or 2 well concentrations.

Cefoxitin Screen: The "Cefoxitin Screen (6µg/ml)" can be used to predict the presence of mecA-mediated resistance in *Staphylococcus aureus.* Isolates for which cefoxitin MIC rests >6 (positive growth) should be reported as oxacillin resistant. Those which have cefoxitin MIC`s of ≤6 (no growth) should be reported as oxacillin susceptible.

QUALITY CONTROL

Sensititre Plates: The inoculum suspension should be cultured onto a suitable medium to check for purity. Test results are invalid if a mixed culture is detected. Subculture frozen or freeze dried stock cultures onto appropriate primary isolation medium and incubate under the appropriate conditions for the organism (primary subculture). Subculture frozen or lyophilized cultures twice before use in testing. The second subculture is referred to as day 1 working culture.

All Sensititre plates include positive control wells. Tests are invalid unless there is distinct growth in all positive control wells. Some plate formats also include a "negative growth" well. This well is used for calibration of the OptiRead and is not required for manual reading. In the unlikely event that growth is observed in the negative growth well, when read on the OptiRead results will not be reported and should not be reported if being read manually.

A number of factors influence MIC determination including organism state, inoculum density, temperature, broth, antimicrobic and culture volume. In practice, replicate MIC's form a normal distribution with the majority of results lying between one dilution of the modal value. At least one of the control organisms listed in the quality control tables is recommended for monitoring the automated 18-24 hour susceptibility test procedure. The test procedure can be considered satisfactory if the susceptibility results obtained with the control organisms are within the expected ranges. Results should not be reported if QC results are outside the stated ranges.

SPECIMEN COLLECTION AND PREPARATION

Specimens should be collected, transported, stored and then plated on to primary isolation medium to give isolated colonies using standard procedure.

Broth

Use only broth supplied by TREK for plates that are to be read automatically.

In addition to routine testing, broth not supplied by Sensititre should be tested for the presence of antagonists to sulphonamides. This may be accomplished by using an organism such as *E. faecalis* ATCC 29212™ and comparing results in the test broth along with those obtained in that broth plus 5% lysed horse blood (LHB). The LHB will neutralize the main antagonists. If there is a significant difference (>2 wells) in the results obtained with and without LHB, then the broth is unsuitable for use in Sensititre plates. Frequency of quality control testing should be established by the individual laboratory in accordance with the laboratory licensing regulation. For further guidelines, refer to CLSI document M7 (26) and VET01 and VET08. Contact Thermo Fisher Microbiology Technical Support for assistance in the event that quality control discrepancies cannot be resolved. See page below for contact information.

EXPECTED VALUES

Expected QC values are provided. For expected values of routinely occurring organisms, it is recommended that each testing site generate a comprehensive antibiogram to determine the percentage of susceptibility to each antimicrobic tested. Antibiotic resistance may vary due to nosocomial infections and geographic location. It has been reported for certain antimicrobics that increased resistance may occur over the life span of the antimicrobic. For full plate information, including plate layout, QC information, Interpretative criteria, performance data and references please refer to www.trekds.com/techinfo. The plate code and batch number will be required.

LIMITATIONS

1. Trained clinical personnel are necessary to make proper interpretations of test results.
2. In common with all other methods of antimicrobic susceptibility testing, the results generated by Sensititre susceptibility plates are *in vitro* results.
3. Sensititre susceptibility plates are configured to meet the CLSI recommendations to detect methicillin-resistant *Staphylococci*. A two per cent salt supplement is therefore included with oxacillin. Inoculum must be prepared directly from an overnight agar plate and not from a fresh broth culture. Be aware that most heteroresistant *Staphylococci* are usually resistant to multiple antimicrobics including ß-lactams, aminoglycosides, macrolides, clindamycin, chloramphenicol and tetracycline and this should be used as a clue to detecting cross-resistance among the penicillinase-resistant penicillins.
4. *Staphylococci* tested against penicillin G should also be tested for ß-lactamase production, especially in strains with borderline MIC's (0.06 to 0.25 µg/ml)
5. The OptiRead should not be used to read nitrofurantoin with *Enterococcus spp*. Nitrofurantoin should be read manually. A nitrocefin ß-lactamase test should be performed to detect ß-lactamase producing strains of *Enterococcus spp.*
6. Poor growth of non-enterococcal strains of *Streptococci* in Mueller-Hinton broth may give unreliable results with aminoglycosides.
7. Broth supplied by TREK has been specially formulated and quality controlled for autoreading Sensititre plates. Broth not supplied by TREK is not recommended for autoreading.
8. Plates should not be incubated in a CO2 incubator except when put up with either *H. somni* or *A. pleuropneumoniae* isolates.

9. The ability of the Sensititre system to detect resistance with *Streptococcus pneumoniae* and vancomycin is unknown because such strains have not been observed. If such a strain is observed, it should be submitted to a reference laboratory.
10. Coagulase negative Staphylococci should be read manually with Sensititre standard veterinary MIC plates unless the plate is designed specifically for Gram positive use only. Most standard veterinary MIC plates are designed for animal species, therefore must be read manually due to lack of signal generation.

PERFORMANCE: Sensititre plates read either manually or automatically and are designed to give comparable performance to CLSI reference micro-broth procedure. Comparable performance is defined as >90% agreement to within a doubling dilution of the reference MIC on a typical mix of organisms

APPENDIX 1: *Colony Count Procedure for Sensititre Plates.*

1. Immediately following the inoculation of the plate, using a 1µl loop, take a sample from the positive growth control well and streak it onto a blood agar.
2. Take a fresh loop (1µl) and sample from the same growth well and mix with 50µl sterile deionized water. Streak a loop (1µl) of this dilution onto a blood agar plate to obtain countable colonies.
3. Incubate both plates at 34 –36 °C over night under appropriate conditions.

APPENDIX 2**:** *MIC Results for ESBL Confirmation*

To confirm ESBLs a >3 twofold concentration decrease in an MIC for either the antimicrobial agent tested in combination with clavulanic acid versus its MIC when tested alone = ESBL. Example: ceftazidime MIC = 8µg/ml ceftazidime/clavulanic acid MIC = 1 µg/ml. For more details refer to CLSI M7
The ESBL confirmatory test was developed for clinical isolates and has not been validated for veterinary isolates

DISCLAIMER

The information provided in this technical insert is current at the time of printing and may change without notice.
The latest information can be downloaded from www.trekds.com\techinfo or by Contacting Thermo Fisher Scientific Microbiology Technical Support

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