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From birth to resistance: characterising calves in 4 dairy farms from Northern Portugal

Do nascimento à resistência: caraterização de vitelos em 4 explorações leiteiras do Norte de Portugal

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Do nascimento à resistência: caraterização de vitelos em 4 explorações leiteiras do Norte de Portugal

Área científica: Saúde Pública

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RESUMO

Reconhecendo o papel do médico veterinário na saúde pública e a resistência antimicrobiana na saúde global, é fundamental dirigir esforços no sentido de perceber como atenuar as preocupantes previsões futuras desta problemática que ameaça o objetivo de uma saúde sustentável e equilibrada para pessoas, animais e ecossistemas, postulado no conceito *One Health*. A consciencialização europeia e mundial sobre esta temática, a par dos recentes avanços nas tecnologias de sequenciação genómica, que acompanham a crescente monitorização da produtividade e saúde das explorações de animais de produção, propiciam a criação de evidência científica rigorosa que possa orientar os procedimentos a adotar no setor pecuário de modo a contribuir para travar a resistência antimicrobiana no nosso meio. Este trabalho reflete o estudo de quatro explorações leiteiras na zona Norte de Portugal desde um panorama geral até aos animais mais novos, pretendendo-se nestes compreender a sua saúde à luz de uma monitorização externa, avaliando o seu ambiente e crescimento, e de um conhecimento da respetiva imunidade adquirida e microbiota, conectando tal informação com o consumo de antibióticos na exploração e os genes de resistência presentes nas fezes daqueles que se incluem na etapa que está descrita com a maior prevalência de resistências antimicrobianas na exploração leiteira: a recria de vitelos.

Palavras-Chave: Resistência Antimicrobiana; Exploração Leiteira; Recria de Vitelos; Sequenciação Genómica

ABSTRACT

Recognizing the role of veterinarians in public health and antimicrobial resistance in global health, it is essential to direct efforts towards understanding how to mitigate the concerning future forecasts of this threat, which highlights the goal of sustainable and balanced health for people, animals, and ecosystems, as postulated in the One Health concept. European and global awareness on this topic, along with recent advances in genomic sequencing technologies, which accompany the increasing monitoring of productivity and health in livestock farms, facilitate the creation of rigorous scientific evidence that can guide the procedures to be adopted in the livestock sector in order to help curb antimicrobial resistance in our environment. This work reflects the study of four dairy farms in the northern region of Portugal, from a general overview to the youngest animals, aiming to understand their health in light of external monitoring, assessing their environment and growth, and understanding their acquired immunity and microbiota, connecting this information with the consumption of antibiotics on the farm and the resistance genes present in the feaces of those included in the stage described with the highest prevalence of antimicrobial resistance in dairy farming: the rearing of calves.

Keywords: Antimicrobial Resistance; Dairy Farm; Rearing of Calves; Genomic Sequencing

INTERNSHIP REPORT

The completion of this article was carried out in parallel with the respective curricular internship, leading to the master's degree in veterinary medicine at ICBAS. The internship period included an initial phase, during which planning was carried out for the visits to the farms, as well as the animals to be selected and the respective harvests. At this stage, the logistical outlines, the necessary materials, and the ethical and scientific framework for the study to be conducted were defined. In the next phase, all necessary samples were collected during successive weekly visits, along with monitoring the health status of individual calves and the practices and indices of each of the 4 farms. Subsequently, after proper storage and preparation of the samples following the recommended protocols, Nanopore genomic sequencing was performed at CIBIO using appropriate bioinformatics tools to process the information obtained for the 95 barcodes corresponding to 43 fecal samples from different animals. In this way, it was possible to obtain for each barcode the present bacteria as well as the antimicrobial resistance genes found, so that statistical treatment and the preparation of illustrative elements could be carried out.

FARM	Health Scoring evaluations	Body measurements	Blood Brix measures	Individuals sampled (Feaces)	Processed samples (Feaces)
FARM1	102	102	30	11	25
FARM2	85	85	37	14	28
FARM3	91	91	29	9	21
FARM4	111	111	43	9	21
TOTAL	**389**	**389**	**139**	**43**	**95**

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ABBREVIATIONS

°C Celsius degree

AMC Antimicrobial Consumption

AMR Antimicrobial Resistance

AMU Antimicrobial Usage

ANIMUSE WOAH Animal Antimicrobial Use Global Database

ARG Antimicrobial Resistance Genes

CIA Clinical Important Antibiotics

DGAV Direção-Geral da Alimentação e Veterinária (Portuguese Veterinary Authority)

DNA deoxyribonucleic acid

EC European Commission

ECDC European Centre for Disease Prevention and Control

EFSA European Food Safety Authority

EMA European Medicines Agency

ESVAC European Surveillance of Veterinary Antimicrobial Consumption

EU European Union

FAO Food and Agriculture Organization

FPIT Failure of Passive Immunity Transfer

g gram

GISSA Quadripartite Global Integrated System for Surveillance of Antimicrobial Resistance and Antimicrobial Usage

GLASS WHO Global Antimicrobial Resistance and Use Surveillance System

h hour

JIACRA Joint Interagency Antimicrobial Consumption and Resistance Analysis

Kg Kilogram

L liter

mg milligram

MSs Member States

n number in a group

NCD neonatal calf diarrhea

PCU Population Correction Unit

TPI Transfer Passive Immunity

W Week

WHO World Health Organization

WOAH World Organization for Animal Health

1. INTRODUCTION

1.1. Antibiotics: A Legacy of Discovery and the Threat of Resistance

1.1.1. History and Importance

Antimicrobial medications play a crucial role in disease treatment and are vital for safeguarding the health of both humans and animals, saving millions of lives and contributing to the provision of food at levels that were impossible before (Laxminarayan et al., 2016). Encompassing antibiotics, antivirals, antifungals, and antiparasitics, the accessibility and appropriate application of antimicrobials are crucial for the food chain in plant and animal production (FAO, 2021) treating infections and preventing disease outbreaks in livestock, particularly in intensive farming systems (Van Boeckel et al., 2019) thus enabling the increased needs of a growing global population in a wide and effective production system to assure food security across the globe (Kelbrick et al., 2023).

Antibiotics have probably existed within the microbial world for millions of years being used by microbes as part of their strategy to prevail and evolve among competing microbial species (Spagnolo et al., 2021). Nevertheless, it was only with Alexander Fleming's accidental discovery of penicillin, a natural antibiotic derived from a fungus, in 1928, that antibiotics gained popularity among human society (Lobanovska & Pilla, 2017). Fleming's work and Waksman's work on secondary metabolites production of Streptomyces genus, discovering antibiotics like neomycin and streptomycin, the first agent effective against tuberculosis (Hutchings et al., 2019), connected ancient practices of using natural extracts for healing infections, such as moldy bread, with the "golden era" of antibiotic discovery. This period started following World War II when numerous antibiotic classes that continued to be used nowadays were identified (Muteeb et al., 2023) leading to a reduced mortality rate from infectious diseases per 100,000 inhabitants from 797 to 36 during the 20th century (Armstrong et al., 1999). Fleming's fabulous work was awarded a Nobel Prize, during which the laureate took the opportunity to highlight right away the potential downside of his discoveries in antibiotic therapy, the antibiotic resistance: "It is not difficult to make microbes resistant to penicillin in the laboratory by exposing them to concentrations not sufficient to kill them, and the same thing has occasionally happened in the body" (de la Fuente-Nunez, 2019).

1.1.2. A Natural Evolutionary Battle

When a new antibiotic is discovered in medicine, there is already genetic competence in nature to resist it. This resistance exists not only in the microbial species that produces it but also in all other bacteria that guarantee its survival in a certain ecological niche that is occupied by microorganisms that produce antibiotics (Iskandar et al., 2022) as most resistance genes identified in

pathogenic microorganisms are identical or very similar to the genes extracted from the soil resistome (Aminov, 2009; Forsberg et al., 2012). In fact, from the time diverse antibiotics were discovered by humans and put into clinical usage, resistance was frequently observed evolving to high frequencies in short timespans, usually within a decade or two (Spagnolo et al., 2021). By the 1970s, there was already a scarcity of treatment options that continues to this day since the supply of new, widely known antibiotics quickly ran out (Fernandes & Martens, 2017). The discovery of new antibiotics accompanied by the simultaneous identification of bacterial variants resistant to antibiotics and the clinical pipeline or the lack of newly licensed antibiotics isn't sufficient to address the growing problem of antimicrobial resistance's establishment and spread (WHO, 2021). There are two key explanations for the decrease in novel antibiotics over the last half-century: increasing difficulty in identifying new classes of medications, accounting that the majority of antibiotics identified since then are modifications of current classes, presenting limited novel mechanisms of action, and the skyrocketing costs of innovation development comparing to other therapeutical approaches (Fair & Tor, 2014; Muteeb et al., 2023).

1.1.3. Classification of Antimicrobial Resistance

In the scientific literature, antibiotic resistance can be classified in various ways based on different criteria, such as: the origin of resistance, which can be classified as intrinsic or extrinsic, depending on whether the resistance is natural or developed through genetic mutations/horizontal transfer (Palma et al., 2020); the mode of transmission, which can occur vertically, through cell division from one generation to successive ones, or horizontally, being acquired from other bacteria in phenomena of genetic material exchange (conjugation, transduction, and transformation) (Tao et al., 2022; Von Wintersdorff et al., 2016); and the respective mechanisms of resistance, whether through modification of molecular targets, enzymatic inactivation, efflux pumps, or decreased permeability (Reygaert, 2018). The classification of resistances based on their origin allows, on one hand, to understand the relationship between bacteria and antibiotics and their respective resistance persistence in natural evolution, as well as to associate the evolution of the microbiota external factors modulating the acquisition of new resistances (Oliveira et al., 2024). Thus, we can define natural (intrinsic) resistances as characteristics of a particular species, genus, or bacterial family, in which their mechanisms did not develop in response to selective pressure exerted by antibiotics and are not capable of being transferred horizontally between bacterial cells (Baym et al., 2016; Olivares et al., 2013). The absence of a cell wall or the production of β-lactamases which prevents the action of β-lactams, on microorganisms such as *Mycoplasma sp* and *Klebsiella sp*, respectively (Balsalobre et al., 2019) are examples of these resistances. By nature, the action of each antimicrobial is defined by these intrinsic properties of microorganisms that, in the same way, can circumvent the activity of the

compound by developing extrinsic resistances, implying the existence of a genetic alteration, whether a mutation in the bacterium's genome (only transmissible vertically to its offspring) or through the acquisition of genes in a specific biome (such as the intestine, soil, water, etc.). This acquisition via horizontal gene transfer can occur by genetic information sharing through plasmids or transposons (conjugation), through infection by phages (transduction, or by the uptake of free DNA resulting from the lysis of cohabiting bacterial cells (transformation) in a certain biome (Von Wintersdorff et al., 2016).

1.1.4. Antimicrobial Resistance, a global threat

Antimicrobial Resistance (AMR) has been acknowledged to be one of the top three major public health threats by the World Health Organization (WHO) (Salam et al., 2023) as more than 700,000 people die from antibiotic resistance-associated disease every year and the number is predicted to rise dramatically if radical actions are not taken (O'Neill et al., 2016). Only in 2019, it is estimated that bacterial AMR was directly responsible for 1.27 million global deaths contributing to 4.95 million deaths and predictions say AMR-related deaths can reach up to 10 million deaths annually by 2050 (Murray et al., 2022). AMR poses a threat to livelihoods for millions of people who raise animals for sustenance and 24 million more people—many of whom living in low-income nations—may be pushed into extreme poverty in just ten years as a result of AMR, increasing the prevalence of malnutrition and hunger particularly in countries with weak or insufficient antimicrobial usage rules, regulatory oversight and monitoring systems (FAO, 2024). In fact, the most frequently cited pathway for AMR spillover between human and food-producing animal bacterial populations is through AMR bacteria (and genetic material) in food chain through direct animal contact, handling or ingestion of food contaminated, most of which are colonists of the host gastrointestinal tract (FAO, 2016). These bacteria might be commensal to animals but pathogenic to people, or commensal in both species, but they may subsequently give the human gut resistance to food-borne infections inclusively forming a reservoir of resistance genes in commensal bacterial flora that can be passed between bacterial species (Singer et al., 2003). In accordance with the “One Health” concept, it’s critical to understand the mobility risk of Antimicrobial Resistance Genes (ARGs), as well as their transmission routes to minimize its dissemination across people, animals and the environment (Berendonk et al., 2015).

1.1.4.1. European Policies and Regulation

The European Commission (EC) adopted an Action Plan to tackle AMR on 29 June 2017, supported by a One Health strategy that takes on animal and human bacterial resistance. Enhancing cross-sectoral work, improving the management and prevention of infection, advocating the responsible use of antimicrobials through antimicrobial stewardship, and combining surveillance of antimicrobial consumption and antimicrobial resistance are a few examples of EU actions addressed

to Member States (ECDC, 2024).

The fight against AMR in livestock requires an integrated approach on a global scale that considers economic, cultural and regulatory aspects, involving coordinated measures between human, animal, and environmental health. At European level, the European Union (EU) has been implementing various policies to combat AMR in line with the 'One Health' strategy: from the ban on the use of antibiotics as growth additives and certain antimicrobials in animal feed, under Regulation (EC) 1831/2003, to the ban on antimicrobials of critical importance for human medicine in production animals (Regulation (EU) 2019/6) and the regulation of veterinary prescription of medicated feeding (Regulation (EU) 2019/4), part of the New European Veterinary Medicines Regulation that came into force on 28th January 2022. Concerning Regulation EU 2019/6, it is important to highlight Article 107, which specifies that antimicrobial medicines should be neither used for prophylaxis, applied routinely or used to compensate for poor hygiene, inadequate animal husbandry, lack of care or to compensate for poor farm management, and that the use for metaphylaxis should be restricted when the risk of spreading an infectious disease in the group of animals is high and when there are no other suitable alternatives.

In addition to legislative measures and directives for the implementation of National Action Plans by Member States in liaison with the Commission, the promotion of good practice guides for the use of antimicrobials in livestock farming, including guidelines for veterinarians and producers (EC, 2017) and support to the transformation into healthy and sustainable agrifood systems (RENOFARM), as well as the implementation of monitoring and reporting programs to track the use of antimicrobials and the prevalence of AMR within the framework of the European Surveillance of Veterinary Antimicrobial Consumption (ESVAC) project, represent important initiatives in this European effort against AMR. Wider analyses, such as the Joint Report on Consumption of Antimicrobial Agents and AMR in Animals, Food, and Humans by Joint Interagency Antimicrobial Consumption and Resistance Analysis (JIACRA), produced by European Centre for Disease Prevention and Control (ECDC), European Food Safety Authority (EFSA) and European Medicines Agency (EMA) under a One Health approach on a regular basis, are also performed using data on AMR collected by the EU MSs and compiled in the EUSR (EU Summary Report) on AMR. The JIACRA study focuses on combinations of antimicrobials and bacterial species deemed to be significant for public health to offer an evidence-based examination of any potential associations between AMC and AMR in humans and food-producing animals (ECDC, 2024)

All these guidelines at European level are in accordance with the Farm to Fork Strategy, adopted by the European Commission in May 2020, which aims to be a tool to help shape the EU's path towards sustainable food systems and which includes the objective of reducing by 50% of the

overall EU sales of antibiotics for farmed animals and aquaculture by 2030 (EC, 2020). In 2024, the FAO created the International FAO Antimicrobial Resistance Monitoring (InFARM), an IT platform to support countries in analysing AMR monitoring and surveillance in farm animals and their food products, integrating this data with the WHO Global Antimicrobial Resistance and Use Surveillance System (GLASS) and the WOAH Animal Antimicrobial USE Global Database (ANIMUSE) into the Quadripartite Global Integrated System for Surveillance of Antimicrobial Resistance and Antimicrobial Usage (GISSA) (FAO, 2024).

1.1.4.2. Monitoring of antimicrobial resistance

Specific and ongoing data collection, analysis, and reporting are required for AMR monitoring of commensal and zoonotic bacteria in food-producing animals and respective products. Along with providing pertinent risk assessment data and supporting in the evaluation of targeted interventions, it makes it possible to comprehend the emergence and diffusion of resistance, track temporal trends in the occurrence and distribution of AMR, and identify new or specific resistance patterns (EC, 2017). AMR in food-producing animals is monitored in domestically produced animal populations representing various production kinds to generate data that can be coupled with data on antibiotic exposure. The Commission Implementing Decision (EU) 2020/1729, which is in effect from 2021 to 2027, mandates that AMR should be monitored *in Salmonella* spp*., Campylobacter coli, Campylobacter jejuni* and indicator commensal *E. coli* in the major domestically produced animal populations and the meat derived from them. Additionally*, E. coli* and *Salmonella* isolates that exhibit resistance to extended-spectrum cephalosporins and carbapenems must be further characterized. In cattle, it is mandatory to monitor AMR in individuals less than one year of age by collecting samples of cecal content in slaughterhouses, following a biennial rotation system. Bacteria targeted for this monitoring include: Salmonella spp., commensal indicator Escherichia coli and Salmonella spp. and E. coli producing extended-spectrum β-lactamases (ESBL), AmpC β-lactamases (AmpC), Carbapenemases (CP). Critically important antimicrobials (CIAs) and other compounds crucial to human health are included in the harmonised panels of antimicrobials used for *Salmonella*, *Campylobacter*, and indicator commensal *E. coli.* These panels may additionally bring greater understanding on the resistance processes at play (EFSA, 2024). The European proposed indicators for AMR in food-producing animals samples include resistance to three or more antimicrobial classes, full susceptibility to a predefined panel of antimicrobials and resistance to ciprofloxacin in E. coli besides the proportion of samples containing ESBL-/AmpC producing E. coli. For all areas, the chosen indicators, which should be reviewed at least every five years, are likely to be useful instruments tracking antimicrobial usage and AMR (ECDC, 2017).

1.1.4.3. Antimicrobial Usage (AMU)

According to recent studies, most AMR bacteria in cattle tend to have their origins in AMU in livestock, whereas most AMR bacteria in humans appear to have their origins in AMU in humans. Most of the time, there was a correlation found in humans and animals alike between the amount of antibiotics consumed and the frequency of resistance in the affected microbial populations (FAO, 2016). Surveillance and research are essential to guide stakeholder decisions on how best to slow the emergence and spread of AMR for the good of food security and global health (FAO, 2021). For food-producing animals, the European proposed indicators for antimicrobial consumption are overall sales of veterinary antimicrobials; sales of 3rd- and 4th-generation cephalosporins; sales of quinolones (specifying the proportion of fluoroquinolones); and sales of polymyxins, all measured in milligrams per population correction unit (mg/PCU) (ECDC, 2017). It is used the Population Correction Unit (PCU), a theoretical unit of measurement developed by the European Medicines Agency (EMA) in 2009, adopted throughout Europe, which corresponds to the standardized average weight in kilograms (kg) of all animals at the time of antibiotic treatment (216 kg for cattle in Portugal), multiplied by the population of the respective animal in the country over the course of a year, allowing for comparative estimates (DGAV, 2022). Despite modest increases in sales in 2014 and 2020, the overall aggregated sales for the 25 countries participating in ESVAC reached the lowest ever reported values in 2022, having declined 53.0% since 2011 (from 161.2 mg/PCU to 75.8 mg/PCU). In comparison to 2021 (86.5 mg/PCU), sales in 2022 decreased by 12.4% (EMA, 2023).

In 2019, to give guidance to the use of AM, the European Medicines Agency (EMA) updated the 2014 Categorisation of the Antibiotic Use (EMA/AMEG, 2014) that suggested a three categories classification using the Critically Important Antimicrobials (CIA) in WHO's CIA list. The 2019 revision suggests four categories, Avoid, Restrict, Caution, Prudence; considering a bigger list of antibiotics and the availability of alternative antibiotics in veterinary medicine aiming to decision-making by veterinarians on which antibiotic to use and reduce the spread of antibiotic resistance, also ranking the route of administration impact on antibiotic resistance. The Category A antibiotics should not be used in food-producing animals, Category B includes quinolones, third- and fourth-generation cephalosporins and polymyxins that are critically important in human medicine which their use in animals should be restricted and Category C should be considered only when there are no proper antibiotics in Category D, which encompasses antibiotics that should be used as first-line treatments (EMA/AMEG, 2019).

1.2. Calf Health Management

The preweaning phase of dairy calves signifies a crucial stage in the life of the newborn cattle,

with the first 21 days carrying the highest risk of mortality due to the major susceptibility to disease (Todd et al., 2018). Although calf rearing is a non-productive stage of the animals' life and does not yield immediate financial benefits, it is important to see it as more than an additional expense. Rather, it ought to be regarded as an investment in the farm's future productivity from the first hours of a newborn (da Silva & Noordhuizen, 2013). In fact, the health status of a preweaned dairy heifer can lead to significant economic losses for the farmer, both immediately, due to mortality or medical treatments, and greatly affecting lifelong production, including reproductive efficiency, growth and milk production (N. Urie et al., 2018).The focus of herd health should be on preventing disease therefore giving dairy producers solid herd health advice together with ongoing monitoring and analysis is crucial to understand the best management practices in a dairy farm (Windeyer et al., 2014) and has a significant potential to lower AMU on the farm by preventing illness and so minimizing the need for antibiotics, as well as by cutting expenses and enhancing animal welfare (Hyde et al., 2019).

1.2.1. Housing

The way calves organize themselves into complex social groups in natural conditions (Whalin et al., 2021) highlights the importance of the social status they develop within the group, which influences, for example, individual access to food (Bica et al., 2019).

The housing of calves can be done in barns, outdoors, or in a combination of both systems and it should protect them from extreme weather conditions, ensure adequate ventilation, and provide a dry and comfortable place for the animal to lie down, so that it does not become a limiting factor for the growth of the calves or harm their health (Costa et al., 2016). The most common practice in dairy calf housing, according to data from the United States and Europe separates calves from the dam after birth, spending the first months of their lives being raised individually easing the monitoring of parameters such as milk consumption and avoiding behaviours as cross suckling (Ede et al., 2022). The form of housing (individual or group pens), the cleanliness of the surroundings and facilities, and the direct contact of calves are all elements related to housing that play a significant impact in the spread of diseases, which seems to be higher in group-housed calves especially in larger groups, when compared to individual housing (Breen et al., 2023). However, group housing allows calves to express natural behaviours improving calf welfare and may improve feed intake and weight gain (Costa et al., 2016; Miller-Cushon & DeVries, 2016). It has been found that calves housed in group pens can express their natural activities such as competition with other calves, playing, jumping, and running, indicating enhanced calf welfare (Chua et al., 2002). As an alternative, pair housing reduces some risks associated with large groups without harming calf behaviour (Chua et al., 2002).

Adapting the most suitable method to each farm is important, for example, to ensure

adequate colostrum intake, early disease detection, and lower infection risk. Individual housing for the first two weeks is recommended (Nikkhah & Alimirzaei, 2022b) however it's important to consider the various variables that can impact illness risk, such as the quantity of milk provided, group size and bedding management. Since the comparison between both systems is not unanimous (Costa et al., 2016), keeping calves away from environmental stressors like cold weather by keeping all bedding dry and at the proper depth should be a first priority. An ideal bedding item needs to possess optimal absorbent properties thus long size wheat straw could be ideal for calves of all ages as bedding (Nikkhah & Alimirzaei, 2022a)

It is also important to remember that proper ventilation helps get rid of harmful gasses, excessive humidity, and airborne particles that can spread diseases or make individuals sick (Bonizzi et al., 2022; Halbach & Robertson, 2021), with evidence showing that artificial ventilation is more conducive to the occurrence of disease than natural ventilation (N. Urie et al., 2018).

1.2.2. Peri-partum

The management of the cow's diet and health during the dry season has been linked to the incidence of dystocia, reported as the most common cause of perinatal mortality (S. M. Gulliksen et al., 2008) and is correlated with the newborn's later digestive and respiratory morbidity (Lombard et al., 2007), as well as the levels of IgG in its colostrum, which demonstrates the significance of this stage in the calves' health. (Fischer-Tlustos et al., 2021). Shortly before the expected date of calving, the cows should be transferred to the maternity ward which usually farms do not either have or use properly. Since this is the fetus's first encounter with the outside of the mother's uterus, it should be as clean and well-designed as possible with a high-quality bed and the calves should be moved as soon as possible to prevent problems like those associated with the umbilical scar (Lorenz et al., 2011; Mee, 2008).

At the beginning of the drying period, cows can be vaccinated for prophylaxis against main agents that threaten calves immediately after birth (Rotavirus, Coronavirus, and E. coli), triggering an immune response that results in the presence of antibodies addressed to these agents in the dam colostrum (Durel et al., 2017). Recently, a new vaccination against *Cryptosporidium parvum* (Gp40 antigen vaccine) has been developed (Timmermans et al., 2024). Vaccination in calves can be equally important considering the interval between the decrease of maternally acquired immunity and the increase of immunity developed by the animal. Isolating calves from pathogen exposure and implementing robust biosecurity measures might boost protection through maternal immunity, thereby extending the period until vaccination is required (Chase et al., 2008).

1.2.3. Calf nutrition

Calves are not born functional ruminants. The development of the digestive system occurs after birth in three consecutive phases: Pre-ruminant phase (2 to 3 weeks of age), when the calf mainly consumes milk or milk replacer; Transitional phase, when the calf starts to eat some starter feed and Ruminant phase, which starts with the weaning (Drackley, 2008)

These dynamic changes require careful adjustments in farming practices to enhance their development. A restrictive diet, consisting of only one daily meal, may cause issues in the development of the abomasum and immune system weaknesses (van der Burgt & Hepple, 2013), in that way and according to EU Directive 2008/119/EC on the minimum standards for the protection of calves, calves need to be fed at least twice-a-day up to six months of age. Unrestricted access to clean water from birth should also not be ignored since drinking water reaches the rumen and aids in its development, and promotes higher intakes of starter concentrates, milk consumption, and growth efficiency (Palczynski et al., 2020). Regarding milk, the recommended amount at the peak of intake is around 15% of the calf's live weight, to meet all its growth needs until weaning (Teagasc Irish Agriculture and Food Development Authority, 2017). After reaching the peak milk intake, its consumption should gradually decrease as the intake of concentrate increases so its birth weight is doubled by the weaning age (Akins, 2016). While maintaining milk consumption, consuming more than one kilogram of concentrate per day for three consecutive days ensures they can maintain the growth rate after weaning (Palczynski et al., 2020; N. J. Urie et al., 2018a). Since weaning is an adaptive process with associated stress, it should not overlap other disturbing practices for the animal, such as dehorning, given the increased associated mortality rate (Barry et al., 2020).

It is important to mention that milk and water should continue to be administered to calves in cases of neonatal diarrhea as they constitute an essential source of energy and fluids for their recovery (Lorenz, 2021) and that milk discarded from the farm, whether due to mastitis or AM residues, should not be offered to calves due to the transmission of diseases and the exposure of the gastrointestinal microbiota to the selection of antimicrobial resistances (Lorenz, 2021; Palczynski et al., 2020). The routine cleaning and disinfection of feeding and watering equipment is often underestimated; yet, these materials serve as an optimal culture medium for bacteria due to their water and nutrient content thus, if its cleaning is not proceeded, the infection pressure on the calves will increase (Heinemann et al., 2020).

1.2.4. Colostrum

Colostrum is the milk generated by mammals during the initial days of lactation, specifically the first 48 hours, and it serves as the primary source of nutrients required for growth (Puppel et al.,

2020) and newborns' development (Kaplan et al., 2022) besides providing high energy required for calves thermoregulation (Vermorel et al., 1983). Colostrum carries numerous immune-related molecules and growth factors, such as Igs, lactoferrin, oligosaccharides, lysozymes and epidermal growth factor (EGF) (Playford & Weiser, 2021), having a potential for usage in a variety of treatments for gastrointestinal disorders and illnesses since it inhibits the growth of pathogenic microorganisms while promoting beneficial microbiota (Fischer et al., 2018; Gomes et al., 2021).

A particular emphasis should be taken regarding the greater IgG concentration compared to mature milk, which is an essential component that affects whether calves receive sufficient passive immunity from colostrum (Fischer-Tlustos et al., 2021; McGrath et al., 2016). The cotyledonary synepitheliochorial bovine placenta inhibits transfer of maternal antibodies into foetal circulation thus newborn dairy calves rely on timely ingestion of high-quality colostrum with low bacterial contamination to transfer passive immunity (TPI) as well as for nutritive and bioactive factors (Lopez & Heinrichs, 2022) to obtain an effective immune response against pathogens in their first few weeks of life (Lago et al., 2018). Calves that receive adequate amounts of good-quality colostrum within the first 24 hours of birth will generally, have better health and growth in the pre-weaning stage compared to calves with an inadequate uptake of colostrum (Elsohaby et al., 2015; Furman-Fratczak et al., 2011; S. M. Godden et al., 2019; N. J. Urie et al., 2018b).

Quality of colostrum, usually expressed as the concentration of IgG in colostrum (Godden et al., 2019), varies significantly among cows (Meganck et al., 2014) and depends on many factors, such as breed (Kessler et al., 2020), dam diseases (Arslan et al., 2021), continuous milking (Rastani et al., 2005; Verweij et al., 2014), season of calving (S. Gulliksen et al., 2008), time between calving and first milking (Conneely et al., 2013). Beyond the quality of the colostrum, an insufficient quantity of ingested colostrum could lead to an inadequate uptake of immunoglobulins and feeding a calf too late interfere with the intestinal absorption of dam antibodies whose most optimal is within two hours of age because of the process of gut closure (Robbers et al., 2021; Weaver et al., 2000). After 6 hours of life, it is found that the intestine has already lost about 50% of its capacity to absorb these molecules, at 8 hours after birth, it has 33% of its absorptive capacity and at 24 hours of life, the intestine usually no longer has any capacity to absorb Ig (Cortese, 2009). It is also recommended to milk a cow completely and as quickly as possible after parturition because the antibody concentration starts to decrease after calving and with every milking (Sandra M. Godden et al., 2019; Morin et al., 2010). Feeding 4 L of first-milking colostrum within 2 h of birth, followed by an additional 2 L fed 6–12 h later, is a common practice in the industry. Since very few calves will voluntarily drink 6 L of colostrum, the use of an oesophageal tube feeder for delivery is a used practice, although it can have associated risks (Sandra M. Godden et al., 2019; Urday et al., 2008). When the colostrum administration is not possible just

after milking dam, it is recommended to store colostrum refrigerated until 48 hours, avoiding bacterial contamination at room temperature that can increase numbers of total bacteria associated with higher risk of disease and it may also lead to decreased absorption of colostral IgG by the newborn calf (Gelsinger et al., 2015). For storing colostrum for a longer time while maintaining the immunoglobulin content and reducing bacterial growth, freezing and thawing of colostrum is recommended (Balthazar et al., 2015; Holloway et al., 2001). The presence of bacteria in colostrum - Escherichia coli, Salmonella enteritidis, Mycoplasma bovis, and Mycobacterium avium ssp. paratuberculosis - decreases the absorption of IgG, as they can block the uptake and transport of this protein in the intestinal lumen (Godden, 2008).

In fact, in a study by Morril et al. (2012) using samples of fresh, refrigerated, and frozen colostrum, only 39.4% of the colostrum samples showed satisfactory values for administration, with the remaining being inadequate for feeding either due to high bacterial counts or insufficient IgG, putting the animals at risk of contracting bacterial infections and/or transfer failure, recommending that colostrum should be fed fresh or frozen immediately. In order to promote the reduction of bacterial contamination in colostrum, pasteurization can be a good contribution (Armengol & Fraile, 2020; Godden et al., 2003) (Anderson 2008; Zou et al. 2017) and should be done at 60°C for 60 minutes, preserving the characteristics of the colostrum and the activity of IgG (Godden et al., 2012). In a recent study, it was concluded that, although the level of IgG was not affected by pasteurization, it influenced the absorption of colostrum components and therefore, the passive transfer of immunity (Ganz et al., 2021) just as the administration of pasteurized milk and colostrum in the first 21 days of life was associated with reduction of morbidity throughout the rearing process, a higher body weight at first calving, and increased milk production (Armengol & Fraile, 2020), proving the advantages of implementing this process in the farm. As the gut microbiome plays an essential role during the neonatal period, it is important to develop studies on its association with colostrum preservation processes, such as acidification and pasteurization (Hennessy et al., 2023).

In cases where there is an absence of colostrum of adequate quality, colostrum substitutes can be used, whose efficacy has already been scientifically demonstrated (Lago et al., 2018), providing acceptable serum IgG concentrations (S. M. Godden et al., 2019).

1.2.4.1 Transfer of Passive Immunity

Failure of neonatal calves to absorb immunoglobulin G from colostrum is referred to as Failure of Passive Immunity Transfer (FPIT) and has been linked to increased morbidity and mortality, reduced growth rates, a higher likelihood of culling, and lower milk production during the first lactation affecting the survival, health, and productivity of dairy heifers (Elsohaby et al., 2015; Lombard et al.,

2020; Robbers et al., 2021).

Colostral IgG concentration is an important factor that affects whether calves receive sufficient passive immunity from colostrum (Godden et al., 2012). In order to objectively predict the colostrum quality, it is recommended to measure colostral IgG content indirectly by using a colostrometer (50 g IgG/l = density of 1,045) or a brix refractometer (50 g IgG/l > 22 Brix) (S. M. Godden et al., 2019) or directly by a cow-side immunoassay kit (single line = IgG concentration >50 g IgG/l) (Meganck et al., 2014). Brix refractometry uses light refraction in a specific solution to estimate total protein (Morrill et al., 2015) and the Brix percentage represents the percentage of total solids in the sample, which has a great correlation with IgG (Pisello et al., 2021), not being affected by colostrum or environment temperature as opposed to colostrometer (Elsohaby et al., 2015). Consequently, it may not accurately predict first-milking colostrum IgG concentration but %Brix scores of 19.0, 22.0, 25.0 and 30.0 percent can be used to estimate minimum colostral IgG concentrations of 25, 50, 75 and 100 g/L, respectively (Mugnier et al., 2020), where the 50 g/L threshold can be considered the standard for first-milking colostrum (Ahmann et al., 2021; Uyama et al., 2022). According to Buczinski & Vandeweerd (2016), a Brix result ≥22% could be used to define good quality colostrum and could help decide which colostrum to keep when using a frozen colostrum bank while Brix value <18% is compatible with low quality colostrum and specific action should be taken if using this sample for calf feeding (such as using colostrum replacer, higher volumes, or colostrum supplements). If the colostrum Brix is between 18% and 22% it may be considered as suspect, and adding frozen colostrum or colostrum supplement is recommended.

To determine the immunity status of neonatal calves, the historical gold standard is a direct measurement of serum IgG concentration using radial immunodiffusion (RID), however it is lengthy, expensive and not practical so cost-effective methods assessing serum total protein concentration are more used and considered a good proxy of IgG serum concentration (Elsohaby et al., 2015; Hernandez et al., 2016; Tyler et al., 1999). Since most of the serum protein in young calves is IgG, both optical and digital refractometers represent reliable ways to measure serum total protein to estimate the uptake of IgG. The fact that refractometry can be applied to serum harvested without centrifugation from blood samples that have been let to clot (Wallace et al., 2006) reinforces the practicality of both refractometers under field conditions. Comparing optical and digital refractometers in the FTPI detection with 52g/L and 8,4% as optimal cut-off values as described in the literature (Mc Beath et al., 1971; Elsohaby et al., 2015, Hernandez et al., 2016, Cuttance et al., 2017; Elsohaby et al., 2019a), no significant difference was evidenced between the proportion of calves classified as having FTPI (Mugnier et al., 2020). However, it should be noted that some discrepancies can be associated with optical refractometer's use since it is susceptible to human reading errors on the measuring scale.

Subsequently, as presented by Lombard et al (2020), using a more elongated categorization of serum IgG concentrations (excellent, good, fair and poor) may allow a more extensive association between TPI and the associated risks (Table 1).

Proposed Categories	Proposed IgG Levels	Proposed % Calves	Equivalent Serum Total Protein Levels	Equivalent Serum Brix Levels
Excellent	> 25,0 g/L	>40%	> 6,2 g/dL	>9,4 %
Good	18,0 - 24, 9 g/L	~30%	5,8 -6,1 g/dL	8,9 - 9,3%
Fair	10,0 - 17,9 g/L	~20%	5,1 - 5,7 g/dL	8,1 - 8,8%
Poor	< 10,0 g/L	<10%	<5,1 g/dL	<8,1%

Table 1. Categorisation of colostrum according to four parameters: IgG levels, % of calves, equivalent serum total protein levels, equivalent serum Brix levels (adapted from Lombard et al. 2020)

Due to the variations in colostrum quality, being conscious of the quality of the colostrum demands good control. In that way, it can be useful to teach the farmer how to make a simple measure of colostrum quality by using refractometry (da Silva & Noordhuizen, 2013). Knowing the quality, increasing the volume of administration can be an option to compensate lower quality colostrum to avoid inadequate transfer of passive immunity (Robbers et al., 2021). Implementing a quality colostrum feeding program is an easy variable to control on the farm that can lead to a decrease in morbidity and mortality.

1.2.5 Mortality and Morbility

Avoiding illness and mortality is one of the major goals of a dairy farm, especially when disease leads to death leaving major economic impact and consequences in performance in later life, evidenced, for example by a smaller body weight associated with individuals that present diarrhea (Windeyer et al., 2014) or pneumonia (Stanton et al., 2012). In addition to being an indicator of animal health, mortality is an indicator of animal welfare (Roche et al., 2020). Although reported mortality rates vary greatly by age, passive transfer status, type of operation, housing, season, nutrition, hygiene and origin of the data set, enteritis and pneumonia emerge as the most common reasons for disease-related deaths among dairy calves and heifers (Curtis et al., 2016; Sivula et al., 1996; Svensson et al., 2006; Tyler et al., 1999; USDA, 2016). In calves aged <31 days, enteritis is the most common cause of death with a case fatality risk for NCD (neonatal calf diarrhea) of 4.9% and a peak probability of dying due to enteritis during the second week of life (Svensson et al., 2006; Windeyer et al., 2014) and pneumonia is the most important problem in replacement heifers over 30 days of age (Agerholm et al., 1993; Svensson et al., 2006; Virtala et al., 1996). Although the cattle industry has made great improvements with herd management, animal facilities and care, feeding and nutrition, and timely use of biopharmaceutics, calf diarrhea and pneumonia are still

problematic due to the multi-factorial nature of the diseases (Cho & Yoon, 2014).

1.3. Intestinal microbiota and resistance genes

1.3.1 Microbiota in Calves

The gastrointestinal microbiota plays an important role in maintaining host health by providing vitamins and energy (Pham et al., 2021), by facilitating development of gut tissue, immune system and modulating inflammatory responses (Macpherson & Harris, 2004) and by competing with pathogens for nutrients and attachment sites on the gut epithelial surface (Pickard et al., 2017). The initial development of gut microbiota correlates with growth in pre-weaned calves, playing a crucial role in optimal calf performance post-weaning, as it has been suggested to enhance the profitability of the cattle industry (Malmuthuge & Guan, 2017). Although traditional focus has been on individual pathogens on calf diarrhea, it has become increasingly clear in diverse species that microbial populations play a key role in health and disease. Disruption of this ecosystem, otherwise known as "dysbiosis," can trigger harming gastrointestinal disorders (Litvak et al., 2017). To date, the molecular basis of dysbiosis and the key bacterial groups involved remain poorly defined however it is clear that if the gut microbiota is disrupted, for example due to antibiotic treatment or gut inflammation, the risk of disease can increase substantially (Gomez et al., 2017; Shin et al., 2015), same as the re-establishment of the normal microbiota can result in disease recovery (Van Nood et al., 2013).

Young calves undergo dynamic changes in their intestinal bacterial community during the first 12 weeks of life in parallel with metabolic and physiological development in the transition from a monogastric to a ruminant animal since an early age (Uyeno et al., 2010). The intestinal bacterial community may reflect such development, concurrent with the establishment of anaerobic conditions in the calf's digestive tract. For instance, strict anaerobes such as those of *Bacteroidales* become dominant in the large intestine, and this corresponds with a decline in the number of facultative anaerobic organisms (Du et al., 2023).

1.3.2 Antimicrobial Resistance Genes in Calf Faeces

The reservoirs of ARGs present in food-producing animals commonly confer resistance to clinically important antibiotics (Wichmann et al., 2014).
The identification of ARGs in the faeces of cattle that were not exposed to antibiotics shows that AMR is an old phenomena that does not just arise from the use of antibiotics (Liu et al., 2016; Morley et al., 2011). This demonstrates that ARGs may be chosen and enhanced by co-selection of resistance genes or by choosing certain microbiome components that harbour these resistance genes, all without the need for the right antimicrobial. Diverse ARGs may be widely distributed and maintained in nature due

to their co-localization in complex resistance clusters, which include genes coding for either antibiotic, metal, and biocide resistance (Amarasekara et al., 2023; Gillings et al., 2017; Pal et al., 2015).

Antibiotic-resistant bacteria, including Salmonella species and Escherichia coli, are often age-dependent in dairy calves, with a larger number in the pre-weaned calf stage of life, however this trend is not always attributed to recent antimicrobial usage (Liu et al., 2019). Environment, diet, cattle source, management practices and farm location have been identified as confounding factors in previous attempts to ascertain the relationship between antibiotic use and the prevalence of AMR genes and its spread and risk to human health (Manyi-Loh et al., 2018; Zhuang et al., 2021). The possibility that the host genetics may affect both the gut microbiome and the profiles of microbial-associated resistome in the digestive tract of livestock and other species is emerging (Liu et al., 2019), as found in the study of T.Ma et al, (2021) showing a less diverse resistome in crossbreed cattle. The same study revealed that besides the breed factor, age may also affect how antimicrobials affect the resistome as the gut microbiota in young animals may be less resistant to outside disturbances and resistome is more likely to shift in response to early antimicrobial usage (T. Ma et al., 2021). Farms practices such as use of cattle manure, usually recycled to agricultural fields, also represents a potential path and a selection pressure of ARGs from manure to soils, ground water, and food systems (Marti et al., 2013) irrespective of the antibiotic use at the origin (Wichmann et al., 2014). In fact, ARG-carrying bacteria in the fecal microbiome have a higher chance of contaminating the surrounding environment, even though the microbial communities of all the dairy cattle's GIT compartments are significant (Liu et al., 2016). Additionally, in dam's dry period, antibiotic treatment was associated with altered microbial composition throughout lactation and high abundances of ARGs in milk compared to the non-antibiotic control group, while using teat sealant without antibiotics led to a reduced abundance of mastitis-causing pathogens (Patangia et al., 2023). Likewise, the colostrum administered must be taken into account since it is strongly possible that it acts as a vector for ARGs that are transferred to dairy calves as stated in the study by Liu et al. (2019) in which the dominant taxa in colostrum were taxa expected to contain high levels of ARGs and around 90% of the ARGs in the dairy fecal samples from day two were also found in the colostrum resistome.

Antibiotic-resistant bacteria are generally more common and abundant in young animals than in adults (Gaire et al., 2020), therefore, it is crucial to comprehend the early acquisition and extent of AMR in calves during nursing to lower the abundance of ARGs. Examining the dynamics of ARGs in the production chain of several dairy farms, Salerno et al (2022) discovered that throughout the whole production chain, the ARG load reduces and that calves' group had more ARGs than the other categories, both for each tested ARG and even for their overall abundance with the sampling age category, rather than the sampled farm, being the significant driver; hence, the abundance of ARGs in

cows' guts might be influenced by factors linked to their early diet (Dill-McFarland et al., 2019). This outcome is consistent with the findings of Liu et al. (2019), who identified colostrum as the primary source of ARGs in the calf's gut. Liu et al. (2019) also found that dairy calves had an overall reduction in the abundance of ARGs as they aged, which pattern was directly correlated with the decrease in relative abundance of specific microbial taxa, for example, Enterobacteriaceae, predicted to harbor the most ARGs, during early life. The correlated reduction of both ARGs and specific taxa is promising because it suggests that interventions targeting the reduction of these taxa may further reduce the ARG reservoir in the gut of cattle. This possibility is supported by other studies like Hu et al (2016) who found that the resistome is mainly structured by the bacterial phylogeny, and that Proteobacteria (which includes Enterobacteriaceae) were more likely to carry resistance than other.

The mobility and potential for propagation of ARGs are mostly determined by gene location thus the most significant way that ARGs propagate across different habitats and among hosts is through horizontal transfer between phylogenetically distant bacteria, which is made possible by ARGs on plasmids and ICEs (Sørensen et al., 2005). Studies have revealed that ARGs may serve additional purposes outside AMR transmission, affecting the host and the microbiota in the digestive tracts of food-producing animals. For instance, certain AMR components, like efflux pumps, may control the microbiota's metabolism of amino acids, fatty acids, or nucleotides while also providing inherent resistance to antibiotics (Aminov, 2009). Separating ARGs that particularly contribute to the spread of AMR from those that might have an incidental effect in microbiome processes that affect host productivity could be a future objective (Tao Ma et al., 2021). Furthermore, it is evident that people, companion animals and farm animals can all acquire ARGs between each other. Most farms have companion animals like dogs and cats, which allows ARGs to spread between them and other species (Tao Ma et al., 2021). In most studies, feaces were used to investigate the impact of antimicrobials on the diversity, profile and abundance of ARGs within the resistome however it should be noted that while fecal samples are easy to collect and are often used as a proxy of the microbial population within the digestive tract, composition of the microbiota differs across segments within the digestive tract (Salaheen et al., 2021; Stanley et al., 2015) Additionally, compared to faecal microbiota, which originates from digesta, mucosa-associated microbiota can directly interact with the host thus mucosa-associated microbiota are more likely to contact antimicrobials within the blood stream as shown by Levesque et al (2012).

1.3.3. Genomic sequencing

Traditionally, two state-of-the-art approaches have been used to detect ARGs within their bacterial host. One approach is based on culturing, antimicrobial susceptibility testing, and polymerase

chain reaction. However, this approach only applies to culturable bacteria and does not enable the discovery of distantly related or unknown elements (Founou et al., 2016) The chance of sequencing genes in organisms that reacted to antibiotic selection and showed functional responses can be increased by using the conventional enrichment technique followed by sequencing. This strategy is comparable to nature, where low seed populations are not problematic until they are increased by antibiotics or coselection. By enhancing the recovery of high-quality long DNA segments and focusing sequencing on DNA of interest, the enrichment stage enhances sequencing results, lowers sequencing costs, and allows for the evaluation of functional samples (Qian et al., 2021). Whole genome sequencing is another approach for detecting complete genomes in bacteria, including ARGs, yet it may not reflect the complexity of the resistome in vivo as a diverse and ever-changing bacterial community is present in both human and animal digestive tracts (Ho et al., 2020) New Genomics Technologies (NGS) have accelerated environmental microbiome research using shotgun metagenomics, allowing analysis of whole genomes and identification of unknown genetic elements, offering the advantage of examining the entire microbiome community and resistome in culturable or unculturable samples and expanding the databases of microbial genomes that are available (Allen, 2014; Li et al., 2018; Thanner et al., 2016). Despite the promise for AMR surveillance, shotgun metagenomics may be underreported due to sequencing depth limitations, particularly for genes that tend not to be prevalent (Burcham et al., 2019). Risk assessment is made easier by third-generation sequencing, which can pinpoint the flanking genes, genomic position, and host range of ARGs. Comparing Nanopore and PacBio to short-read sequencing techniques, the primarily mentioned provide high alignment coverage, filter nonfunctional ARGs, and provide high confidence in ARG identification, particularly in plasmid assembly (Qian et al., 2021).

Mobility and host context determine ARG risk, and MGEs such as plasmids, transposons, and ICEs are important for HGT propagation, which presents a greater danger to public health. Given that these genes are more likely to be transmitted to pathogens and compromise therapeutic therapy, it is also suitable to particularly research the transferable ARGs (Zankari et al., 2012) Indeed, Liu et al. (2019) found that the diversity of transferable ARGs increased with time, counteracting the declining abundance of total ARGs. The accumulation of additional species during the assembly of communities is partially responsible for the increasing variety of transferable ARGs. Therefore, the existence of ARGs does not imply that they are expressed or capable of conferring AMR. Since variations in gene expression and regulation may impact phenotypic resistance, Forslund et al. (2013) suggested using the word "resistance potential" rather than "resistance.", consequently the starting point in integrating metagenomic techniques into AMR surveillance in the agricultural sector should be characterizing the "resistance potential" of environmental and animal-derived samples.

2. FIELD WORK

2.1. Objectives

This experimental study, conducted in four dairy farms in the Northern Zone of Portugal, aimed to characterize the health status, microbiome and resistome of calves monitored during the practical work period. Alongside the monitoring of the calves, the aim was to record data and practices of the farm that could be related to the characterization of the individuals under study, including colostrum practices, housing, and the consumption of antimicrobials.

2.2. Material and methods

2.2.1. Sample collection

Framed in the Project USAM SuLei, four dairy farms in the North of Portugal were randomly selected and assigned a code from FARM1 to FARM4 to maintain confidentiality. The animals included in the study included all the calf's born during the study period, except those not expected to be kept in the farm in a minimum period of 3 months, thus the sample comprised mainly Holstein Friesian females.

The data was collected from calves' birth until they were 8 weeks old. The 8 weeks will further be referred as Week 1, W1 = 0 to 7 days, Week 2, W2 = 8 to 14 days, Week 3, W3 = 15 to 21 days, Week 4, W4 = 22 to 28 days, Week 5, W5 = 29 to 35 days, Week 6, W6 = 36 to 42 days, Week 7, W7 = 43 to 49 days, Week 8, W8 = 50 to 56 days.

2.2.2. Morbility and mortality

The animal's health status to evaluate respiratory and diarrhea morbility was assessed using the Calf Health Scoring Chart from the University of Wisconsin-Madison: Four-level scoring scale for the diagnosis of diarrhea based on fecal, where 0 = normal consistency, 1 = semi-formed or pasty, 2 = loose feaces and 3 = watery feaces (calves were considered positive for diarrhea when the fecal score was > 2) and for the diagnosis of Respiratory Disease, also using 4-level scoring scales with nasal discharge (0 = normal serous discharge, 1 = small amount of unilateral cloudy discharge, 2 = bilateral cloudy or excessive mucus discharge, and 3 = copious bilateral mucopurulent discharge), ocular discharge (0 = normal, 1 = small amount of ocular discharge, 2 = moderate amount of bilateral ocular discharge, and 3 = heavy ocular discharge), coughing (0 = none, 1 = induced single cough, 2 = repeated coughs or occasional induced spontaneous coughs, and 3 = repeated spontaneous coughs), ear position (0 = normal, 1 = ear flick or head shake, 2 = slight unilateral droop, and 3 = head tilt or bilateral droop) and rectal temperature (0 = 37.8 to 38.2°C, 1 = 38.3 to 38.8°C, 2 = 38.9 to 39.4°C, and 3 = . 39.4°C). Calves were considered positive for diarrhea when the fecal score was > 2 and considered

positive for respiratory disease when the aggregate score was >5.

All criteria parameters were recorded individually during weekly monitoring visits of the calves under study, using a standard thermometer (±0.1 Celsius) to assess temperature. Verification of all births and departures (due to sale or death) from the farm were checked every week.

2.2.3. Growth

A total of 93 holstein calves were observed after birth and 69 were followed minimum in their first two months of life. In order to monitor the calves' growth, weekly measurements were always made by the same operator using a measuring tape (±0.1 cm), for a total of 10 weeks. (W1: first week from birth to 7 days, W2: from 7 to 14 days; W3: from 14 to 21 days; W4: from 21 to 28 days; W5: from 28 to 35 days; W6: from 35 to 42 days: W7: from 42 to 49 days; W8: from 49 to 56 days; W9: from 56 to 63 days; W10: from 63 to 70 days).

393 measures of Heart Girth (HG) and Body Length (BL) were taken to estimate the body weight (BW). Thoracic circumferences were accessed by the HG and the BL was measured from the distance between withers height to the top of the tail head. Later, the body weight (BW) was estimated based on the Heinrichs et al. (1992) study, deducing the BW from the HG, since among the features evaluated in the mentioned study HG had the highest correlation and minor associated error. One farm (FARM3) was testing a weighting balance and the measured values were used for comparison and validation of the manually taken measures.

2.2.4. Colostrum

To evaluate the colostrum administration for each individual, it was requested that at the time of colostrum's administration, the responsible farmer recorded the respective information on the colostrum administration method, Brix measurement of the colostrum administered (using the same refractometer model with weekly calibrations), postpartum hours of administration and quantity administered in the first dose of colostrum. To assess the acquired passive immunity, the total proteins in the serum were measured using a Brix refractometer in the first 7 days of the animal's life by collecting blood samples via the coccygeal vein into clean dry tubes.

2.2.5. Antimicrobial consumption

For the evaluation of antimicrobial consumption at the level of the calves under study, producers were asked to record the medications administered during the rearing phase in each farm, which was monitored during weekly visits and verified by consulting the respective veterinary prescriptions. Evaluation of antimicrobial consumption at the farm level was assessed by analyzing

farms' invoices between January 2024 and July 2024 to include the study period.

2.2.6. Microbiota and Resistance genes

The evaluation of the fecal microbiota of the animals under study (n=45) included the collection of feaces from calves from the four farms at three different periods of the animal's life: the first week, the first month and at the end of two months of life. After collection into the indicated tubes, the fecal samples were stored in packages with dry ice for up to 6 hours, until storage at -80C.

Subsequently, the stool samples were subjected to DNA degradation using the MagMax Microbiome Ultra Nucleic Acid Isolation Kit protocol and subsequently, the DNA was sequenced using the Native Barcoding Kit 96 V14 protocol. For the characterization of the microbiota and the quantification of resistance genes EPI2ME workflow "Fastq Antimicrobial Resistance" from nanopore was used, performing quality control of the filtered and trimmed reads, taxon identification via the WIMP (What's in My Pot; workflow, based on the NCBI RefSeq database and Centrifuge, and resistance gene identification using the Comprehensive Antibiotic Resistance Database (CARD). WIMP files were imported to R using PACKAGE epi2me.r and converted to a phyloseq object. A total of 5673 taxa were identified. After Standardize abundances to the median sequencing depth using phyloseq package (cita) for better comparison the number of taxa was 404. For diversity assessment between samples and between farms Shannon diversity test was applied. As the samples are non-independent, a linear mixed model was used. A beta diversity model was applied to visualize the differences between the microbiome profiles of the samples through a Principal-Coordinates Analysis (PCoA), from the Bray Curtis dissimilarity matrix, that did not reveal a clear clustering pattern. To check if the microbiome composition was statistically different between groups the vegan package was used to perform a Permutational Multivariate Analysis of Variance (PERMAVONA) analysis based on the Bray-Curtis matrix.

2.3. Results

2.3.1. Evolution of calf population and morbility

Overall, the study calf population decreased in the first 60 days of life (Figure 1A). The decrease represents animal deaths since sold animals were not considered in Figures 1A and 1B. The total number of animals decreased from 69 calves to 59, over the 60 days. The mortality rate resulting from the fraction between the number of dead animals over the number of newborn calves was approximately 14,493%. The biggest losses occurred in the first 10 days with 50% of the deaths occurring in that period. Regarding each farm, FARM1 did not lose any animals in the first 60 days, FARM 2 lost 3 animals until day 20 while FARM3 and FARM4 lost 2 and 3 animals respectively until day

30 (Figure 1B).

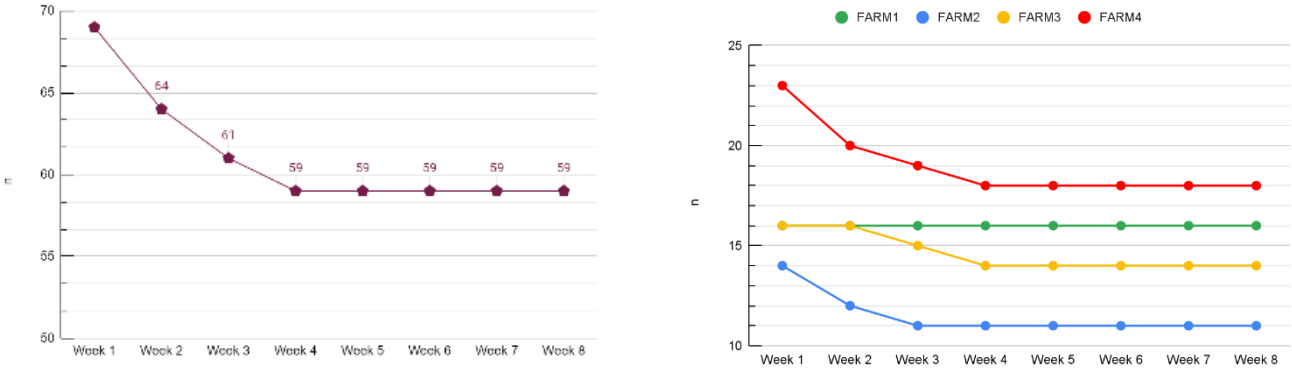


FIGURE 1A; 1B. (A) Evolution of calf herd across the first 6 weeks of life within all farms; **(B)** Population in the first 8 weeks per farm. Sold animals were not considered.

The period with the biggest disease prevalence was observed in the first two weeks of calves' life with 10 sick animals (2 with pneumonia and 8 with diarrhea) reported in the first week and 9 sick animals (2 with pneumonia and 7 with diarrhea) reported in the second week. After this period disease drastically drops, not registering more than 3 sick animals afterwards (ANNEX B). This scenario could either indicate that disease has been controlled over the two first weeks or that the animals would be more susceptible in that period.

2.3.2. Growth

The two measured parameters BL and HG increased gradually through the weeks of study (Table 2) along with the calculated average weight of the studied calves (Figure 2). The average estimated weight of the first week was 41,13 kg and the tenth 73,45 kg (Table 2). FARM3 showed the highest medium value of BW from week 1 (93,68kg) and average weight over the weeks, with its biggest increases occurring between weeks 4 and 5 (0,71 kg/day), and 5 and 6 (0,72kg/day). FARM4 weight values were below the mean of the total studied group during the study period while FARM 1 had the highest daily mean weight gain (0,65 kg/day).

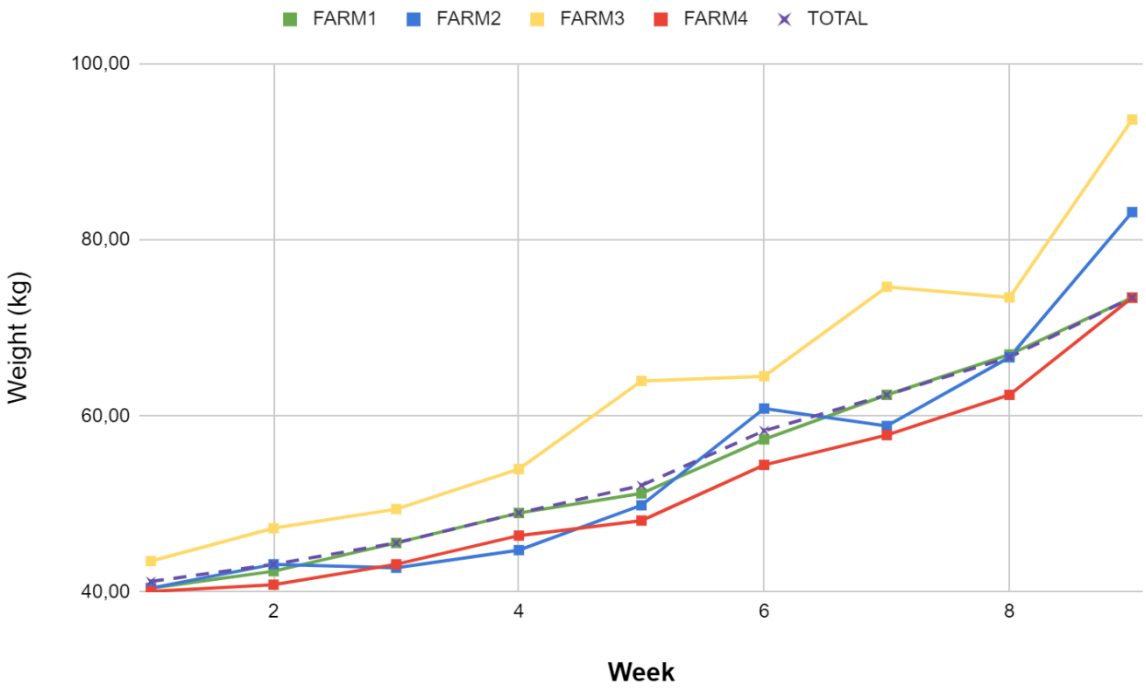


FIGURE 2. Estimated weight evolution per farm.

TABLE 2. Weekly measurements of BL, HG and deduction of BW (kg) = 65.36 − (1.966 × HG) + (0.01959 × HG2) + (0.00001691 × HG3), where HG = heart-girth (cm) (Heinrichs et al. (1992)

Week	n	BL (cm)	HG (cm)	Estimated BW (kg)
W1	82	55,23	80,69	41,13
W2	70	56,41	82,18	43,41
W3	59	57,44	83,70	45,24
W4	51	59,29	86,31	48,54
W5	39	61,10	88,50	53,30
W6	31	63,60	91,71	59,29
W7	28	64,84	93,64	63,45
W8	19	66,89	96,53	67,39
W9	11	68,32	98,15	78,09
W10	3	67,33	99,33	73,45

2.3.3. Colostrum feeding

The four studied farms presented different values for each evaluated category as seen in Table 3. The average colostrum BRIX value varied between 24,00 (FARM3) and 25,88 (FARM2), the average administered colostrum quantity varied in a small range of 3,84 L (FARM3) and 3,34 L (FARM4), with a total average of 3,53 L while average post-partum hours at which colostrum was administered varied between 1,8h (FARM1) and 5,5h (FARM4). The average blood BRIX value varied slightly despite the colostrum BRIX value with FARM3 presenting the highest blood BRIX value (9,07) and FARM2 having the lowest (8,20).

Farm	n	Colostrum BRIX value	±	Quantity (L)	±	Post-partum hours (h)	±	n	Blood BRIX value	±
FARM1	17	24,88	*3,3*	3,50	*0,4*	1,81	*2,1*	11	8,66	*0,8*
FARM2	23	25,88	*1,9*	3,55	*0,7*	2,57	*1,8*	17	8,20	*0,7*
FARM3	19	24,00	*3,1*	3,84	*1,0*	2,97	*3,2*	19	9,07	*0,7*
FARM4	28	25,84	*3,9*	3,34	*0,9*	5,46	*1,2*	19	8,85	*1,1*
Total	**87**	**25,22**	*3,03*	**3,53**	*0,81*	**2,77**	*1,96*	**66**	**8,76**	*0,77*

TABLE 3. Value of average blood brix (obtained by author), quantity, and post-partum hours of the colostrum administration by farm (obtained from farmers).

2.3.4. Passive of Transfer Immunity

A blood BRIX value classified as “excellent” seems to be related to a higher survival rate of calves, presenting a 100% survival rate at 60 days. This is consistent with Figure 3 in which the distribution of blood BRIX value in dead animals is lower. Nevertheless, “weak” blood BRIX value was linked to a higher survival rate (87,5%) than “ok” or “good” colostrum. It can be also observed a higher interval of blood brix levels in individuals which were still alive at day 60 (Figure 4).

Comparing the colostrum BRIX value administered to the study calves based on the dam’s lactation, no relevant difference was found between primiparous (25,17%) and multiparous (25,26%).

The correlation between the two refractometers used was obtained with R^2 =0,899.

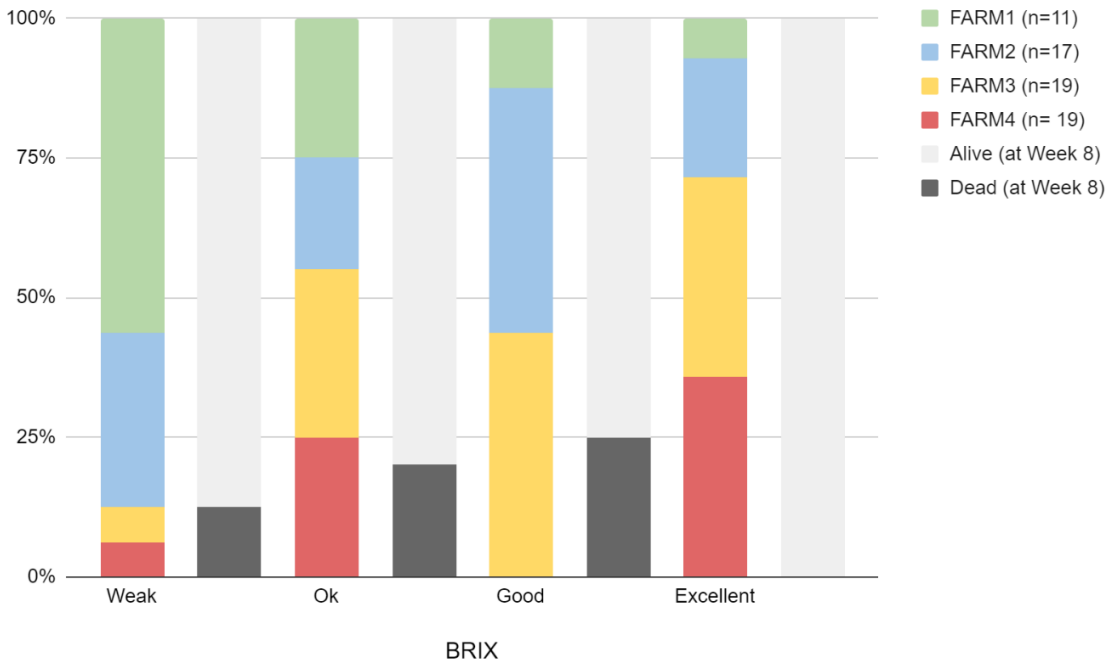


FIGURE 3. Categorized Blood BRIX value by farms and corresponding mortality. Blood BRIX value quality was determined using the following classification: weak (<6); Ok (7,8- 8); Good (8,1-9); Excellent (>9,3).

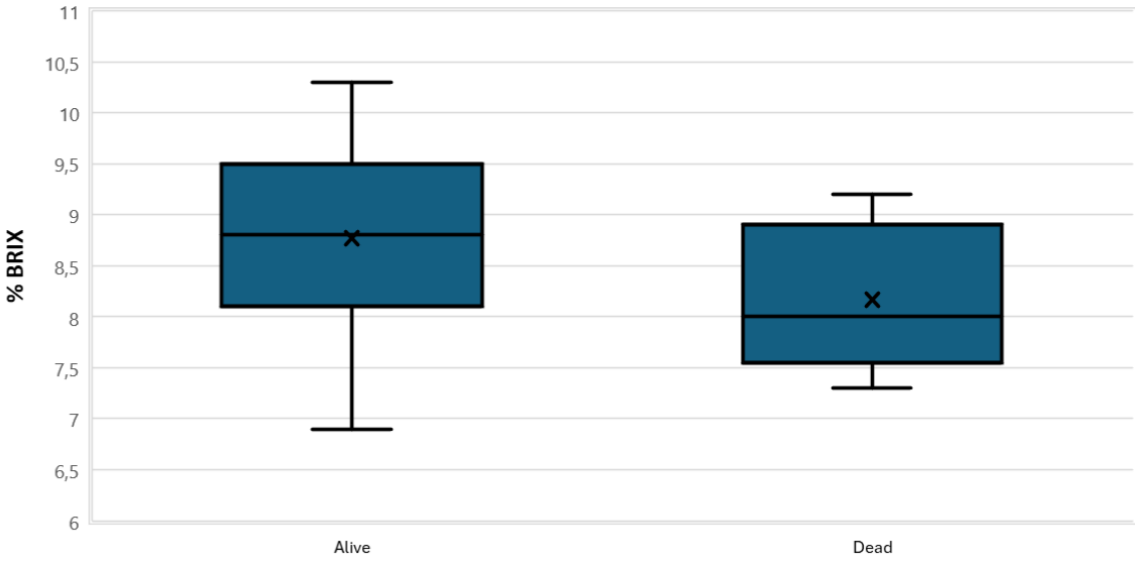


FIGURE 4. Box-plot distribution of Blood brix levels in Alive or Dead animals at day 60.

2.3.5. Antibiotic consumption

14 of the 16 administrations recommended for respiratory syndrome or diarrhea included patients who had a positive score for either illness at the visit before or after the administration (ANNEX B). Additionally, five antibiotic administrations for different medical conditions were noted, including nonspecific hyperthermia (n=2) and omphalitis (n=3). The most widely class of antibiotics administered in study calves was Sulfonamides (41,2%) followed by Macrolide which use was less than half of the previous (17.6%) (Figure 9). In

alignment with that, the most widely used AMEG category of antibiotics was D (52,9%), followed by C (41,2%) as the second most prevalent, and Category B showed up with a small usage (5,9%) while category A was not used in the studied animals (Figure 5A,5B).

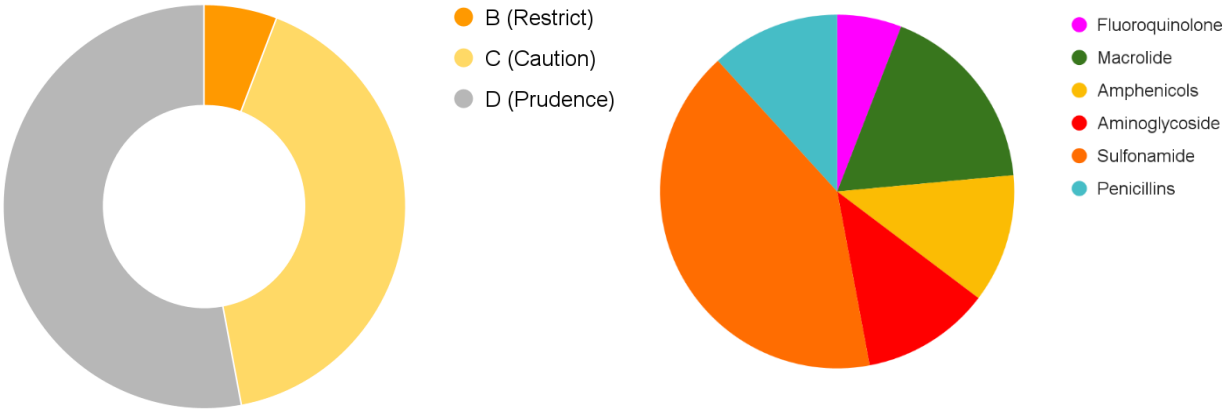


FIGURE 5. (A) Distribution of used antibiotics by classes; (B) Distribution by AMEG categories.

2.3.6. Feaces Microbiota

The results of the fecal sequencing analysis can be accessed through the following link (https://drive.google.com/drive/folders/1s8MtC5I6IybM4UH9I-4JsJK38LZ43jZf?usp=sharing) in order to enable a clear visualization of the figures, being referenced as Supplementary Material (S).

2.3.6.1. Taxonomic and diversity analysis

Taxonomic identification revealed that during the three sampling periods, fewer total taxa were found, and the specific taxa of each sampling decreased parallelly, with maximum total and unique taxa in the first 7 days (Table S1).

Furthermore, shared taxa between sampling periods were maximum between the first and second sampling (124), decreasing between the second and third (93), and were minimum between the first and third harvest (72) (Table S1) suggesting that the faecal microbiota changed more from week 4 to week8 than from week1 to week4 and it kept changing throughout the 60 days. This can be evidenced by a Shannon Index linear mixed model between the intercept (WEEK1) and the fixed effects of WEEK4 and WEEK8 in which the negative correlation demonstrates a reduced diversity across the respective samples. The model also shows that the effect of the sampling week on alpha diversity (Shannon) is significant only in WEEK8 compared to WEEK1 while the change in alpha diversity between WEEK1 and WEEK4 is not significant. The same model was also used to access diversity between farms however no significant differences between farms were found in alpha diversity (Figure

S). The calculated low value of random variance for ANIMAL indicates that variability between animals is not an important source of variation in this context. The lack of substantial dissimilarities in microbiome composition for farms was confirmed by PERMANOVA (pseudo-F = 1.1385; p = 0.246) while the beta diversity plot using principal coordinate analysis (PCoA) based on beta diversity did not evidence clustering patterns among samples based on either farm or sampling.

2.3.6.2. General analysis

Changes in the proportions of bacterial species present in feaces were observed throughout the 3 sampling periods. The most common genus was Clostridium across the 3 sampling periods, followed by *Escherichia* and *Faecalibacterium*. *Aquimarina*, *Bacteroides*, *Collinsella*, and *Lactobacillus* were also present but in much smaller quantities.

At the species level, *Clostridium sensu stricto* is the most represented species, again showing high abundance throughout the different sampling points. Other notable species, such as *Escherichia coli* and *Lactobacillus johnsonii*, become more prominent in the 30-day sample but are reduced in the 60-day sample. *Bacteroides fragilis* is more common in the two first samples than in the third. Notably, the species *Romboutsia timonensis* and *Faecalibacterium prausnitzii* maintain steady but low abundance across all periods (Figure S4-S11).

2.3.6.3. Antibiotic exposure

The faecal samples collected from calves around 7, 30, and 60 days of age reveal notable variations in the composition of the intestinal microbiota, both at the genus and species levels. These variations are further differentiated between the groups exposed and not exposed to antibiotics. Across the sampling periods, both groups show a big predominance of Clostridium (*Clostridum botulinum*). Other relevant present genera are Bacteroides, Rombustia and Bifidobacterium, in the week1, and an increase in the abundance of Lactobacillus in non-exposed samples. By the third sampling, exposed samples exhibit a notable proportion of Nostoc. At the species level, *Clostridium botulinum* emerges as a dominant species in both groups but is particularly prevalent in antibiotic-exposed calves.

2.3.6.4. Farms

The four samples from different farms reveal distinct microbial communities. Clostridium genus, specifically *Clostridium botulinum,* is predominant in all four farms. Escherichia, specifically *Escherichia coli,* are also present in all four farms being the second most prevalent in all, except for FARM4, where Lachnoclostridium predominated, accounting that Clostridium scindens was considered a bacteria from Lachnoclostridium genus by the used software.

Genus like Martella, Aquamarina and Nostoc, Pelosinus, and Megamonas are farm-specific for

FARM3, FARM1, FARM2, and FARM4, respectively. Limosilactobacillus are present in FARM2 and FARM3. Bifidobacterium, especially *Bifidobacterium longum*, is present in all four farms, although with a small abundance. Also, *Lactobacillus johnsonii, Bacteroides fragilis* and *Faecalibacterium prausnitzii* can be found in all four farms with a small abundance.

FARM4 has a big abundance of *Clostridum scindens*, also present in FARM2 with a much smaller prevalence. *Phocaeicola vulgatus* is present in FARM3 and FARM1. *Collinsela aerofaciens* is present in FARM1 and FARM3 (Figure S4-S11).

2.3.7. Resistome

2.3.7.1. Farms

The farms show varying degrees of AMR gene abundance and diversity, with certain genes being more consistently detected than others. The heatmap displays intense red and orange coloring in FARM3, which translates into a greater abundance of resistance genes like tetQ, APH(3')-Ia and tetW, also predominant in FARM2. FARM3 shows a more subdued presence of resistance genes in comparison to FARM1 and FARM2, with lighter shades across most genes except fo tet44 and mefA with higher abundance compared to other farms, while Farm AYM00 exhibits mainly faint yellow shades indicating the lowest abundance and diversity of AMR genes across all three time points compared to the other farms (SM; Figure 21).

2.3.7.2. Sampling

In the first group of samples, with feaces collected from WEEK1 calves, a high-intensity coloration (yellow to red) is observed suggesting that some resistance genes are already established in the microbiome of the calves as early as maximum 7 days. The highest abundant findings are APH(3')-la (dark red), a resistance gene to aminoglycoside antibiotics, such as streptomycin; tetW (orange), tetB(P) (roasted yellow), tetQ (yellow) and tetA (light yellow), conferring resistance to tetracycline. In the second group of samples, the feaces where collected from 30-day-old calves. During this period, there is a noticeable decrease in the intensity of gene detection across many samples, indicated by the predominantly lighter shades of yellow and white. The most abundant genes (roasted yellow) during this period seems to be tetW, tetQ, conferring resistance to tetracycline and ANT(6)-Ib gene that encodes an enzyme known as aminoglycoside 6-nucleotidyltransferase which confers resistance to streptomycin, by modifying the antibiotic through nucleotidylation. In the third group of samples, the feaces where collected from 60-day-old calves. The overall detection of resistance genes seems to be lower with only a few samples showing major detection reflecting the abundance of tetW (red), tetQ (orange) and tet40 (roasted yellow), conferring resistance to

tetracycline and mefA (yellow) which encodes efflux pumps that extrudes macrolide antibiotics (Munita & Arias, 2016).

2.3.7.3. Species

In Table 10 (SM), it is notable that E.Coli was clearly the bacteria harbouring the most ARG genes accounting for 39,81% of the resistome across all the fecal samples analysed. It is also notable the contributions of *M. smegmatis* and *M.tuberculosis*, secretory of PknG (virulence factor), as well as *Bifidbacterium longum*, already demonstrated as health gut promoters and Bacteroides fragilis whose benefits are still discussable. Between all the samples and including all the genes identified and their respective antibiotic resistance, the aminoglycoside (39,15%) and tetracycline (19,94%) resistance were independently the resistances more presented. Although not visible in the heatmaps which were made using the "clinical resistance" filter, peptide (6,88%), fluoroquinolone (4,63%) and aminoccumarin (3,87%) were also classes whose resistance genes were widely identified. Despite the low abundance, the multi-drug resistance to classes like tetracycline antibiotic, disinfecting agents and antiseptics, penem, phenicol antibiotic, rifamycin antibiotic, penam, cephamycin, glycylcycline, cephalosporin, carbapenem, monobactam, fluoroquinolone antibiotic is an important finding.

2.4. Discussion

2.4.1. Calf Health management

2.4.1.1. Mortality and morbility

Dairy calves' preweaning period is an important time in their lives with the first 21 days being the most critical because of the increased vulnerability to illness (Todd et al., 2018). Ideally, there should be no preweaned calves' deaths, but rates of up to 5 % between days 1–60 after birth are considered tolerable (DCHA, 2013). However, many studies have found mortality rates above this threshold (Cuttance et al., 2017; Zucali et al., 2013; Swan et al., 2007), as the present study reported, demonstrating a 14,5% mortality in the first 60 days, of which 11, 6% occurred in the first 21 days. It should be noted that individuals were included in this study in a period between their first 1-7 days, thus mortality before calves were included might be underestimated, taking into account that the average incidence of perinatal mortality (up to 24 h) varies between 2 and 20 % in studies published in different parts of the world, with most studies reporting rates between 5 and 8 % (Mee, 2013). Disparities in death rates reported in various studies may be due to calf and herd management practices or the place of the study (Windeyer et al., 2014), as it can be observed by the variation

between the four studied farms whose heterogeneity can be seen in several of the general parameters collected (ANNEX A).

Risk factors for dairy calf mortality include housing type and hygiene (Gulliksen et al., 2009; Mee, 2008), dystocia and inadequate neonatal care (Bleul, 2011; Gundelach et al., 2009), inadequate colostrum and nutrition management (Donovan et al., 1998; Godden, 2008). The two most common and expensive illnesses that affect calves are digestive and respiratory disorders: when combined, diarrhea and pneumonia cause over 80% of preweaning calves deaths (Windeyer et al., 2014; (Fruscalso et al., 2020) Although it was not possible to attribute direct causality to all deaths reported in the present study, farm workers reported diarrhea as a possible cause in 40% (n=4) of deaths until the first 60 days, and it was possible to confirm a *Cryptosporidium* case *post-mortem*.

The majority of diarrhea cases were identified during the first 2 weeks of neonatal age, as described in other studies showing that either the first (Ok et al, 2009; Bendali et al, 1999; Demir et al, 2019), highly significant in this study, or second week (Bartels et al., 2010; Izzo et al., 2011) of calves' life presents the highest incidence of newborn diarrheal calves cases, which might be worsened in winter months due to a higher occurrence of diarrhea pathogens (Berber et al., 2021). Recent literature reports a higher BRD detection frequency of both disease and pathogens in calves aged 35 days or older (Otten et al., 2024) however given the small sample in this study and since BRD is a multifactorial complex disease associated with environmental, host factors, and multiple pathogens, it was not possible to establish a suggestive relationship between the weeks under study and the highest number of BRD cases. In that way, to avoid or at least lessen the incidence and severity of future outbreaks, it is crucial to identify and comprehend the etiology of digestive and respiratory calves' disorders in order to address the causes and implement appropriate preventative measures against these multifactorial diseases whose environment and management practices can influence severity or outcomes (Cho & Yoon, 2014; Gorden & Plummer, 2010).

2.4.1.2. Growth rate

Growth rate is one of the most important parameters for monitoring calves and heifers' performances from birth to calving (Bazeley, 2016). Compared to Turini L. (2021) results of Holstein heifers' weight estimation, the calculated BW and the measured HG of the current study are under Turini's confidence values for the same period. On the other hand, BL values are smaller than what the literature presents. This might have come from the fact that the measuring methods for the BL were facilitated for the author's convenience (distance between withers height to top of the tail head) since its not always specifically mentioned in the literature, differing in the anatomical measurement locations for length and making comparisons difficult. Therefore, HG, thought to have the strongest association with body weight (Heinrichs et al., 1992; (Heinrichs et al., 2017)) was selected as the

comparison parameter with values previously published in the literature. All measures were taken by the same handler, who used the same reference points every week, decreasing the bias related to the operator. The most significant growth in FARM2 calves, particularly from week 6 onwards, reaching over 100 kg by week 8, can be attributed to several factors, including management practices (Machado & Ballou, 2022), environmental conditions (Bell et al., 2021), nutritional strategies (Ockenden et al., 2023) and microbiota influence (Du et al., 2023) while the diverse weight at the starting point (week 1) may be explained by genetics variances (Ware et al., 2015).

2.4.1.3. Colostrum and passive immunity

To assess the best practices for colostrum feeding in order to meet the newborn calf's demands, the "Three Q's" strategy may be used by the farmers, regarding colostrum's quality, quantity, and quickness administration (Palczynski et al., 2020). Consequently, in terms of quality, a 22% Brix score can be considered an appropriate cut-off value to identify good quality colostrum corresponding to 50g/L on IgG concentration (Bielmann et al., 2010). Concerning quantity and quickness, a minimum 3 litters should be provided with the first feeding (S. M. Godden et al., 2019), using an oesophageal tube when calves do not voluntarily ingest at least 2 litters (Chigerwe et al., 2008). It is recommended to first administrate ideally in the first 2-3 hours (Robbers et al., 2021), while the efficiency of IgG is maximal, and to complete at least 6 litters within the first 24 hours (S. M. Godden et al., 2019). Overall, the studied farms administered the colostrum according to the recommendations, although some late first feeding could occur due to night natural parturitions between the farms affecting the average of each one. The number of daily milkings can also affect the first feeding timing since usually the farmers wait until the routine milking schedule of each farm. In terms of quantity and quality, except FARM4, all the farms use oesophageal tube to guarantee the total consumption and have their refractometer to assess the dam's colostrum quality before voluntary feeding, using colostrum bank or colostrum substitute when the values did not meet the intended values.

Evaluating the transfer of passive immunity according to Lombard et al, (2020), we can see that FARM2 obtained the highest proportion of weak acquired passive immunity while FARM4 and FARM1 the highest proportion of excellent acquired passive immunity. However, since the quantity, quality and quickness (Table 4) are provided by the farm owner and have not been validated by the author, making associations between these and the assessment of animals' serum Brix is not accurate. The premise that higher serum Brix values are associated with lower mortality can easily be seen in Figure 6 where the animals alive at day 60 are associated with higher serum Brix values than those already dead. In fact, it is possible to demonstrate that the animals with an excellent level of passive immunity transfer were all alive on day 60. The assessment of serum total protein was made indirectly

by using two optical refractometers with a g/L and %Brix scale, which values are considered a good IgG serum concentration estimate (Hernandez et al., 2016; Elsohaby et al., 2019a). The comparison between both showed a good correlation with an R^2=0,895.

Concerning the association between estimated immunity in serum and the number of lactations, we can see identical values between primiparous and multiparous cows, highlighting that pregnant dams in their second lactation revealed a higher average brix value than the others, corresponding to 9.08 (n=18), although it has already been described in the literature that the lowest IgG values were found for second-lactation cows (S. M. Gulliksen et al., 2008). The comparison made in this study compares the serum proxy IgG that as stated above it may be directly influenced by each administration in colostrum quality, quantity and post-partum hours feeding making this comparison barely viable, however reviewing the literature it is evident that the dam's parity affects the colostrum's IgG content (Bielmann et al., 2010).

2.4.1.4. Administered antibiotics

The usage of antimicrobials is in line with the national data gathered by DGAV in the 2022 National Report of monitorization of antimicrobial consumption. Regarding Figure 9, we can notice the absence of Tetracicline (most sold group of AB in 2022 in Portugal, representing 33,33%) in used antibiotics in the studied group. According to DGAV report, the 5 most used classes were Tetraciclines, Penicilines, Macrolides, Lincosamines and Fluoroquinolones. In the present study, Sulfonamides, Macrolides, Penicilines, Amphenicols and Aminoglicosides were the most used classes. Interestingly, Tetraciclines (class D antibiotic) were not used in the study group. Regarding Figure 10 and comparing to Portuguese report which shows that the most sold class of antibiotics in 2022 in Portugal was class D Prudent (61%), followed by class C Caution (30%) and B Restrict (9%), it is observed a less use of group B antibiotics but a higher percentage of treatments using antibiotics belonging to the Group C. Comparing the data collected with the national data it should be taken in consideration that collected data refers to consumed antibiotics by the enrolled calves during the work period and national data refers to total sold antibiotics by all authorized wholesale distributors of veterinary medicines covering all veterinary medicines for all different animal species of any age.

2.4.2. Microbiota

2.4.2.1. Sampling

The general findings on microbiota of the studied animals are in agreement with established literature on calf gut microbiota development being Bacillota (Firmicutes), followed by Pseudomonadota (Proteobacteria) and Bacteroidota (Bacteroidetes) the most prevalent phylum

across all samples (Du et al., 2023; Meale et al., 2016; Oikonomou et al., 2013). The dominance of Firmicutes (Oikonomou et al., 2013) and its increasing average prevalence occurred from the first to the fourth week of life, as demonstrated by Du et al (2023). This is consistent with other studies in pre-weaned calves and reflects the common patterns seen in neonatal calves where strict anaerobes, essential for initial gut colonization and digestion of fibres, dominate early microbial development both in rumen and gut (Malmuthuge et al., 2014; Uyeno et al., 2010) supporting the early nutritional needs of the calf as the digestive system matures (Malmuthuge & Guan, 2017). Contrary to the study of Oikonomou (2013), Proteobacteria, whose presence was already evidenced in calves faeces 20 minutes after birth (Guzman et al., 2015), increased from the first to the second month of life in the individuals sampled of the present study.

The genus-level shifts, particularly the increased presence of genus like Escherichia and Faecalibacterium at 30 days, also reported by (Kim et al., 2021), are likely due to changes in housing conditions with environmental exposure and increased calves' interaction between each other, and diet management (Cray et al., 1998). A higher prevalence of Faecalibacterium, one of the major producers of butyrate in the large intestinal (Lopez-Siles et al., 2017) known for its anti-inflammatory properties and already linked to gut health in humans and animals (Mayer et al., 2021) has been associated with lower incidence of diarrhea and higher weight gain in calves during the first weeks of life (Oikonomou et al., 2013), which may reflect a beneficial adaptation in the studied individuals gut as they develop. According to prior research, giving F. prausnitzii orally to nursing Holstein calves appears to inclusively improve intestinal health and development while dramatically lowering the incidence of severe diarrhea and death (Foditsch et al., 2015)

At the species level, the persistence of Clostridium sensu stricto throughout all sampling periods aligns with its known importance in young ruminants, aiding in the cellulose degradation, a crucial process for fiber digestion (Du et al., 2023). In fact, Clostridium is naturally present in cattle's gastrointestinal tract (Peek et al., 2018) but its dominance in all samples, especially in antibiotic-exposed groups, raises concerns about chronic dysbiosis and the potential pathogenicity of certain species that are the primary biomarkers to identify the gut microbial structure disorder and have a stronger colonization following the microbial structure disorder (Redding et al., 2021). *Clostridium botulinum*, which can cause botulism, a potentially fatal disease in humans ((Rawson, A., 2023)), or *Clostridium perfringens*, the major toxin producer, also present in contaminated colostrum, dirt, sewage, water, stored feeds, and housing surroundings (Simpson et al., 2018), are examples present in samples from this study. Of the five *C. perfringens* toxinotypes (A, B, C, D, and E), type C is mostly found and pathogenic in calves less than 10 days, generally younger than 5 days, causing haemorrhagic enteritis and unexpected death in newborn calves (Robi et al., 2024).

Escherichia coli was largely found across all samples which can be expected due to its role in gut colonization shortly after birth (Du et al., 2023), however its persistence varies based on environmental and management factors (Uyeno et al., 2010). Besides serotypes associated to clinical diseases, such as enterotoxigenic E. coli that was not found in this investigation even non-pathogenic E. coli strains can act as secondary infectious agents by multiplying under a dysbiosis of the gut community, leading to systemic problems and death from colisepsis (Gomes et al., 2024). *Mditerraneibacter gnavus* is another species example that, despite its identification and decreasing abundance in feaces of pre-weaning calves which has already been documented (Amin et al., 2023), both negative and positive effects in humans have been linked to it such as the synthesis of pro-inflammatory chemicals (van Soest et al., 2020), and the fermentation of dietary fibers and generation of butyrate (Nilsen et al., 2020). Regarding Lactobacillus species, besides the *L.reuteri* absence in the 2 month sampling, it is evident an abundance decrease across the sampling periods, as a noticeable decrease abundance of *L.johnsonii* in AB non-exposed calves. In fact, this species were already reported a dominant abundance in feaces of healthy calves during the first week of life being associated with less probability of diarrheal disease and a significative reduction 24h before clinical signs in diarrheic calves (Schwaiger et al., 2023) testifying its potential as probiotics (Fernández et al., 2020) and in preventing colonization of pathogens (Fan et al., 2021). Growing research indicates that when taken as probiotic, also Bifidobacteria (with considerably lower presence in AB exposed samples), provide antimicrobial properties which help protect the host gastrointestinal tract, preventing bacterial and viral diarrhea (Servin 2004; Timmerman et al. 2005).

2.4.2.2. Antibiotic exposure

The persistence of Clostridium dominance, coupled with the reduced relative abundance of beneficial genera like Faecalibacterium and Lactobacillus in the different sampling periods in AB exposed calves suggests that the gut microbiota may not fully recover, even after the cessation of antibiotic treatment, noting that this depends on each individual specific context (Ng et al., 2019). In both groups, despite being a usual observation during the early phase of the pre-weaning period since its introduced directly to newborn calves through the feaces of the dam (Kim et al., 2021), the presence of Bacteroides fragilis can be alarming as it is the most virulent species in the genus Bacteroides and some strains can produce an enterotoxin causing diarrhea in animals (Myers et al., 1985). Interestingly, P. vulgatus is present in the first week of life, with more expression in antibiotic exposed samples. In fact, in recovery phase of calf diarrhea that may coincide with the first sampling period in AB exposed calves, Wang et al. (2024) documented a higher relative abundance of P. vulgatus which may potentially influence the growth and health of preweaning calves, corroborating other studies showing that diarrhea is associated with a decrease of Bacteroidetes (Oikonomou et al., 2013). It was also found

a notable presence of Romboutsia, a spore forming bacteria, in exposed calves at the first sampling, which its strong resistance and tolerance to environmental stress or restricted nutrients may be responsible for its microbial development (Lin et al., 2023).

In calves not given antibiotics, the microbiota appears to evolve into a more diverse and balanced community over time preventing pathogen colonization by competitive exclusion, with beneficial genera like Lactobacillus, related to digestion of milk and immune system modulation (Schwaiger K, et al. 2023) and Faecalibacterium, whose abundance has been associated with weight gain and less diarrhea (Oikonomou et al., 2013), becoming more prevalent. *Faecalibacterium prausnitzii,* in particular, contributes significantly to intestinal health by generating butyrate, a short-chain fatty acid with a high energy value that maintains gut barrier integrity and has anti-inflammatory properties (Savin KW, 2019).

The findings suggest that early antibiotic exposure in calves might have a long-term impact on the composition of the gut microbiota suppressing the activity of beneficial bacteria or at least perturbing the community naturally formed in the animal intestine, as reported by Uyeno et al (2010). The observed decrease in microbial diversity is consistent with research indicating that even low antibiotics concentration impair the natural development of a balanced microbiota, causing dysbiosis and allowing opportunistic infections to thrive (Dobrzanska et al., 2020; Yousif et al., 2018). Du Y, et al. (2023), also indicated that giving calves antibiotics early in life may cause a long-lasting decrease in good bacteria and raise their risk of developing metabolic and gastrointestinal issues in later life. Antibiotics may suppress the activity of beneficial bacteria like Lactobacilli, as well as the target pathogens (Collier et al. 2003), or at least perturb the community naturally formed in the animal intestine (Castillo et al. 2006; Tajima et al. 2010). One potential measure other than probiotic cultures to enhance the impact of beneficial bacteria and mitigate the use of antibiotics is the use of oligosaccharides. Nondigestible oligosaccharides have been used in calf diets to eliminate harmful bacteria from the intestine and help improve the animals' health (Heinrichs et al., 2003)

Insights into how the bacterial community in calf feaces changes with age may affect illness prevention and calf health maintenance. Substantial and sudden alterations in the fecal microbiota appear to reflect the GI tract's fluctuating microbiome thus establishing and stabilizing the microbiota from an early stage of growth may be one of the most important conditions for an organ to become functional. In this sense, treating pneumonia or diarrhea in cattle by administering antibiotics at an early age may hinder the development of a desirable community.

2.4.2.3. Diversity

Contrary to other studies (Dill-McFarland et al., 2017; Edrington et al., 2012), the within-sample diversity decreased as animals aged until 2 months however, this may be due to the higher

number of samples from the first week of life thus with more taxa identifications before and after standardizing abundances. It is also important to emphasize that first week samples of each animal were collected randomly between day 0 and day 7 thus a higher diversity between samples could be observed since there is a significant change in the fecal microbiome in the first week of life due to the gradual exposition to birth canal (Klein-Jöbstl et al., 2014), colostrum, salivary microorganisms, the microbiota of the dams (Kim et al., 2021), environment and udder skin (Alipour et al., 2018; Yeoman et al., 2018). This indicates that calf fecal microbiome inoculation may come from several maternal sources and that calf microbiome development may be predicted using the dam's microbiome (Owens et al., 2021).

Assessing Shannon diversity is an important tool since studies indicate that calves with more diverse gut microbiomes in the early stages tend to exhibit better growth performance and overall health (Du et al., 2023). The reduced unique samples at 2-month sampling in this study supports the idea that environmental microbes are continuously passed through the GIT until particular taxa can establish and proliferate, thereby becoming a part of the resident microbiota with increasing abundances of taxa shared between individuals. The overall stability over the 60-day period supports existing research indicating that the microbial community in calves stabilizes as they transition from milk to a more complex diet, which is essential for their development into functional ruminants both in beef and dairy calves (Weese & Jelinski, 2017). Because many taxa characteristic of the adult phase are already present, the severity of the transition in the microbial community that occurs at weaning may be lessened if solid feeds are consumed prior to weaning (Meale et al., 2016). After weaning, the microbiota continues to converge toward a comparable adult microbiome, and significant changes take place between weaning and one year (Dill-McFarland et al., 2017).

In other studies, it was found an association between morbility and microbial diversity. In Oikonomou et al (2013) diarrhea incidence during the first four weeks of the calves' life was associated with a reduction of microbial diversity during the third week of life, as increased fecal microbial diversity after the second week of life was associated with higher weight gain. The same study reports a lower Chao1 index (measure of the overall number of species in a sample) during the third, fourth, and fifth weeks of life in calves with pneumonia yet, the impact of pneumonia on microbial diversity is likely explained by the fact that all cases of pneumonia were identified during the first week of life and were subsequently treated with parenteral injection of broad-spectrum antibiotics (Oikonomou et al., 2013).

It was clear that Escherichia, a facultative anaerobe commonly associated with broad-spectrum ARGs, made up a sizable amount of the microbiota in the early phases of the microbiota in dairy calves. The early development of these bacteria is at least partially due to colostrum feeding, and

their dynamic variations in relative abundance during nursing result in the gradually changed resistome (Liu et al., 2019). The reduction of both ARGs and particular taxa is associated (Rochegüe et al., 2021), which is encouraging because it implies that therapies aimed at reducing these taxa may also decrease the ARG reservoir in cattle's gut. According to Liu et al. (2019), Proteobacteria—which includes Enterobacteriaceae—were more likely than other phyla to possess resistance corroborating that bacterial phylogeny primarily shapes the resistome.

2.4.3. Resistome

2.4.3.1. Sampling

The observed patterns of AMR gene distribution suggest that antimicrobial resistance is prevalent among dairy calves as early as 1 week old, with notable differences between farms and over time. Although in this study the sample group only consisted of calves, it is known that antibiotic-resistant bacteria are often more common and abundant in young animals than in adults thus it is crucial to comprehend the early acquisition and extent of AMR in calves during nursing given that they could be a crucial point of control for lowering AMR in cows (Liu et al., 2019; Rochegüe et al., 2021). The gut microbiome of some of the study's individuals maintained a natural reservoir of ARGs despite not receiving any antibiotic therapy, supporting the idea that a high frequency of AMR is not always linked to recent antibiotic usage (Khachatryan et al., 2004; Thames et al., 2012) although it can be mainly associated with long-term use (Zhang et al., 2022).

The early detection of AMR genes at 1 week is indicative that colostrum might serve as a vehicle for certain bacterial species that proliferate and temporarily survive in the gut microbiome of dairy calves, with colostrum-detected ARGs accounting for 90% of all ARGs found in the feaces of dairy calves in their earliest days, matching the colostrum resistome pattern. A relevant fraction of colostrum microbiota could be colonized by bacteria from a range of habitats, such as the teat surface, milking equipment, dirt, grass, diet, water or air (Quigley et al., 2013) varying from farm location which could presumably explain the difference in resistome abundance between farms and highlights the importance of colostrum and good peripartum hygiene practices. Eliminating antimicrobial-resistant bacteria in raw milk and colostrum can be achieved through methods like heat treatment, microfiltration, centrifugation, and curdling. These methods can be used at the farm level or centralized for farmers. For example, in Belgium, frozen colostrum surplus is collected, transported to a central location, and sterilized by irradiation and can be purchased in frozen or freeze-dried form for farmers (EFSA, 2017).The absence of colostrum in diet is not the only factor contributing to the decline in ARGs over time; the introduction of novel carbon sources into the diet has led to an increase in taxa that have enzymes capable of degrade plant polysaccharides and are actually low in total ARGs (Liu et

al., 2019).

Regarding specific AMR genes, the gene APH3 was the most abundant in the first week sampling, consistent with (Haley et al., 2020) who also showed a higher prevalence of tetQ in calves faeces, an increasing abundant gene that was the most prevalent in 2 month sampling in the present study. TetW, another tetracycline ribosomal protection protein highly present across all the sampling periods, was also emphasized as a prevalent finding both in calves and cow feaces by Haley et al. (2020). Overall, the second sampling showed a less prevalence of resistance genes with reduced prevalence of most identified genes in the first week, as aph3 and tetO, the presence of genes exclusive to WEEK4, such as OXA 85, and the appearance of genes whose abundance increases in WEEK8 like tet44. This could be explained by it being a period of adaptation to the environment, particularly a collective housing, and the solid pre-weaning diet, with less influence from colostrum administration, preceding the third sampling, in which the microbiota, proved to be significantly different from WEEK1, has already been established.

2.4.3.2. Antibiotic resistance

Grouping the genes based on the antibiotic resistance they confer, it is possible to observe a predominance of tetracycline resistance, increasing from the first to the third sampling, for which the detection of the tetq and tetw genes contributed the most. The increase of tetracycline ARGs like tetQ over time was also documented in the study of Liu et al (2019), in which was found a association with Bacteroidaceae community and a possible connection with historical antibiotic use since no antibiotics were given to the animals in the mentioned study. Regarding aminoglycoside resistance predominates, it decreases considerably from the first week to the animal's 2 months of life. Both resistances were already documented as the most frequent in calves faecal samples, in higher presence comparing to cows' samples. In fact, almost 25% of the most prevalent ARGs in calf samples were aminoglycoside resistance genes and nearly half tetracycline resistance genes (Haley et al., 2020). It is also important to highlight the presence of sulfonamide resistance genes present in the first week, as well as the increasing resistance to macrolides and cefamicin throughout the sampling, evidenced by the abundance of the mefA and cfxA genes, respectively. Regarding multidrug resistance, although this is a volatile concept, especially when applied to veterinary medicine (Sweeney et al., 2018), it is noteworthy to mention the cumulative resistance to penems, cephalosporins, and monobactams in the first sample and the presence in all three samples of genes conferring combined resistance to streptogramins, lincosamides and macrolides.

CONCLUSION

To advise on responsible usage and enable the sustainable availability of effective antimicrobial agents, it is crucial to investigate and eventually comprehend the relationship between animal bacterial resistance and the consumption of antimicrobial products. The relationship between animal species, antimicrobial class, and bacterial level, however, is typically difficult to analyse and describe in depth and calls for precise and thorough information on both antimicrobial resistance and antimicrobial consumption. To ascertain, for example, if variations in antimicrobial resistance among animal species can be linked to variations in antimicrobial agent consumption patterns, comprehensive data by species is required. To accomplish all these goals effectively and precisely, data collection at the species level is crucial. Because intervention methods, such restricting or banning the use of specific antimicrobial classes, may only affect specific manufacturing stages, it is also critical to take these stages into account. It is essential that the data be reliable and representative in order to meet these goals. Particularly difficult to evaluate and compare different research because of the variation in the techniques employed to quantify and report AMR. Although phenotypic resistance was reported in most research, interpretation will remain challenging due to the quick development of genotypic approaches, but it may also yield new information. A thorough understanding of the variables influencing the emergence, dissemination, and persistence of AMR in dairy calves is essential for creating antimicrobial stewardship strategies that work. In order to reduce AMR on dairy operations, evidence-based biosecurity, treatment, and management decisions will be influenced by future research to better understand calf physiology and the effect of management variables on AMR. Future research must make more use of cutting-edge methods for resistome investigation, such as long-read sequencing, with an emphasis on ARG expression and MGE's function in the spread of AMR in the guts of various food-producing animals. Such data will be essential for determining the likelihood that ARGs will move from animals used for food to people and for creating practical plans to lessen the damage that ARGs pose to the environment and public health.

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ANNEX A

General information of studied farms

	FARM1	FARM2	FARM3	FARM4
GENERAL INFORMATION				
Year	1987	2019	2000	1989
Total animals (n)	245	230	420	261
In milk (n)	105	100	190	134
Calves (n)	19	20	40	23
Daily production (L/cow/day)	35	41	40	34
Days in lactation	170	186	150	200
Post-partum interval (days)	398	398	420	394
Total Area of the farm (hectars)	17	30	45	35
WELFARE AND HYGIENE				
Welfare score	50	62	59	59
Deaths in 2023 (SNIRA)	27	12	40	9
Bedding (cows)	Sawdust, Mat	Mat	Mat	Sawdust, Mat,
Feaces hygiene	Tractor	Scraper	Scraper	Scraper
CALVES				
Maternity (days before partum)	15	15-21	10	60
Colostrum first feeding (method)	Probe	Probe	Probe	Nursing bottle
Water *ad libitum*	Yes	Yes	Yes	Yes
Supplement	Yes	Yes	No	No
Pasteurization	No	No	No	No
Bedding	Straw	Straw	Straw	Straw

ANNEX B

Diarrhea and Respiratory Disease Report [with Incidence(I) + AB)] and Deaths per week

		FARM1		FARM2		FARM3		FARM4		TOTAL	
		I	AB	I	AB	I	AB	I	AB	I	AB
W1	Diarrhea	2	0	3	2	2	0	1	0	8	2
	Respiratory S.	0	0	1	1	1	0	0	0	2	1
	Deaths	0		2		0		3		5	
W2	Diarrhea	1	0	2	2	2	1	2	0	7	3
	Respiratory S.	0	0	0	0	2	2	0	0	2	2
	Deaths	0		1		0		1		2	
W3	Diarrhea	0	0	2	2	0	0	0	0	2	2
	Respiratory S.	0	0	0	0	1	1	0	0	1	1
	Deaths	0		0		1		1		2	
W4	Diarrhea	0	0	0	0	0	0	0	0	0	0
	Respiratory S.	0	0	1	1	0	0	0	0	1	1
	Deaths	0		0		1		0		1	
W5	Diarrhea	0	0	0	0	0	0	0	0	0	0
	Respiratory S.	0	0	0	0	0	0	0	0	0	0
	Deaths	0		0		0		0		0	
W6	Diarrhea	1	1	0	0	0	0	0	0	1	1
	Respiratory S.	0	0	0	0	2	1	0	0	2	1
	Deaths	0		0		0		0		0	
W7	Diarrhea	1	1	0	0	0	0	0	0	1	1
	Respiratory S.	0	0	0	0	1	1	0	0	1	1
	Deaths	0		0		0		0		0	
W8	Diarrhea	0	0	0	0	0	0	0	0	0	0
	Respiratory S.	0	0	0	0	1	0	0	0	1	0
	Deaths	0		0		0		0		0	
TOTAL	Diarrhea	5	2	7	6	4	1	3	0	19	9
	Respiratory S.	0	0	2	2	8	5	0	0	10	7
										40	16
	Deaths	0		3		2		5		10	

From birth to resistance: characterising calves in 4 dairy farms from Northern Portugal

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