Faculty of Agricultural and Veterinary Sciences - Unesp, Jaboticabal
Faculty of Veterinary Medicine - Ghent University

Exploring the respiratory microbiome and new diagnostic parameters in *Mycoplasma hyopneumoniae*-infected pigs

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Master in Veterinary Medicine

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TÍTULO DA TESE: EXPLORING THE RESPIRATORY MICROBIOME AND NEW DIAGNOSTIC PARAMETERS IN *Mycoplasma hyopneumoniae*-INFECTED PIGS

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DEDICATION

I dedicate this thesis to those mentioned in the acknowledgements' section.

With love K.S.

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LIST OF ABBREVIATIONS

ADWG	Average daily weight gain
AI	Artificial intelligence
BALF	Bronchoalveolar lavage fluid
BLASTP	Protein BLAST
bp	Base pairs
C	Cytosine
ddNTPs	Dideoxynucleotide triphosphates
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
EP	Enzootic pneumonia
G	Guanine
G. parasuis	*Glaesserella parasuis*
H_2O_2	Hydrogen peroxide
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IM	Intramuscular
IMT	Intramuscular temperature
kb	Kilobase
km	Kilometer
LRT	Lower respiratory tract
M. flocculare	*Mycoplasma flocculare*
M. hyopharyngis	*Mycoplasma hyopharyngis*
M. hyopneumoniae	*Mycoplasma hyopneumoniae*
M. hyorhinis	*Mycoplasma hyorhinis*
M. hyosynoviae	*Mycoplasma hyosynoviae*
M. parvum	*Mycoplasma parvum*
M. suis	*Mycoplasma suis*

MLCL	Macroscopic lung consolidated lesion
NCBI	National Center for Biotechnology Information
NGS	Next-generation sequencing
NT	Nasal turbinate
P. multocida	*Pasteurella multocida*
PCR	Polymerase chain reaction
PCV-2	Porcine circovirus 2
pi	Post-infection
PK-15	Porcine kidney cells
A. pleuropneumoniae	*Actinobacillus pleuropneumoniae*
PM	Particulate matter
PRDC	Porcine respiratory disease complex
PRRS	Porcine reproductive and respiratory syndrome
PRRSV	Porcine reproductive and respiratory syndrome virus
rRNA	Ribosomal ribonucleic acid
S. suis	*Streptococcus suis*
sp.	Single unnamed species
swIAV	Swine influenza A virus
TNF	Tumor necrosis factor
URT	Upper respiratory tract
UV	Ultraviolet

APPROVAL OF THE ETHICAL COMMITTEES IN THE USE OF ANIMALS

The study entitled "**Influence of *Mycoplasma hyopneumoniae* natural infection on the respiratory microbiome diversity of finishing pigs**" and included in this thesis under **Chapter 3**, was approved by the Ethics Committee in Animal Use of the Faculty of Agricultural and Veterinary Sciences of the **São Paulo State University**— Jaboticabal, under the Protocol 2032/21.

The study entitled "**Rationalizing the use of common clinical parameters and technological tools to follow up *M. hyopneumoniae* infections in pigs"** and included in this thesis under **Chapter 4**, was approved by the Ethics Committee of the Faculty of Veterinary Medicine and the Faculty of Bioscience Engineering at **Ghent University**, under the protocol EC 2021-062.

PREFACE

Pork is the second most consumed animal protein in the world, and along with the European Union, Brazil also plays an important role in pig production and exports. Yet, in six years, the world population is estimated to reach 8.5 billion people, while pork consumption will reach more than 130 million tons, with an increase of more than 7%. In order to meet this demand, the swine industry needs to be more profitable and efficient, also aiming for a more sustainable production. In addition to the costs of production, losses associated to infectious diseases also contribute to the lower profitability, possibly jeopardizing the viability of the industry. To meet market demands, the number of animals housed per unit is expected to increase, which might compromise the health of the herd, leading to the development of diseases.

Respiratory problems are among the most costly diseases in swine production worldwide, and alongside the porcine respiratory reproductive syndrome virus, *Mycoplasma hyopneumoniae (M. hyopneumoniae)* plays a great role. *Mycoplasma hyopneumoniae* is believed to affect more than 70% of the herds in the world and is characterized by its high morbidity and low mortality. The bacterium is associated with enzootic pneumonia, but also the porcine respiratory disease complex. The infection is restricted to the respiratory system affecting first the cilia of the airways, compromising the clearance system and allowing the pathogen to descend to the lung, where the disease will progress. In the lung, cranioventral consolidation is the most common macroscopic finding. The main clinical sign of the disease is the non-productive cough; however, when other pathogens are associated, other clinical signs like dyspnea, fever, and decreased performance might be evident. As *M. hyopneumoniae* compromises the mucociliary system of the respiratory tract, it gives a chance to other pathogens to multiply such as *Pasteurela multocida*, *Streptococcus suis*, *Glaesserella parasuis*, worsening the disease.

A variety of microorganisms (bacteria, viruses, fungi, etc.) can be found in different regions of the respiratory tract, and these specific microbial communities represent the microbiome. Yet, investigating the differences between the lung microbiome of pigs

infected and non-infected with *M. hyopneumoniae* might be important to understand the interactions between pathogens, especially those related to the respiratory disease complex. Furthermore, knowing how *M. hyopneumoniae* infection affects piglet movement might shed light into the pathogen's effects on pig behavior, information that might be useful for diagnosing diseases, especially if it could serve as early disease indicator.

Summary – Exploring the respiratory microbiome and new diagnostic parameters in *Mycoplasma hyopneumoniae*-infected pigs

The global demand for pork is rising due to population growth, necessitating expansion in the pig industry. However, respiratory diseases, caused by pathogens like PRRS virus, PCV-2, and *Mycoplasma hyopneumoniae*, present significant challenges in pig farming. While advancements in diagnostic technologies like PCR and sequencing have improved our understanding of the respiratory microbiome in pigs, the interaction between the lung microbiome and *Mycoplasma hyopneumoniae* is less clear. Sensors capable of evaluating behavior such as movement are being introduced to the pig industry. These might give additional information on welfare and might even be able to serve as early predictors of disease.

Accordingly, the thesis aimed to investigate the respiratory microbiome of *M. hyopneumoniae*-infected and non-infected pigs. It also aimed to explore new tools to support diagnosis of *M. hyopneumoniae*. Specific objectives included comparing microbe composition in the upper and lower respiratory tract of infected vs. non-infected pigs and assessing *M. hyopneumoniae*'s impact on piglet movement and body temperature using camera monitors and thermal microchips. Additionally, associations between movement, temperature, and established diagnostic parameters like cough severity, lung lesions, and bacterial load were examined.

Chapter 3 shows that infected pigs had a reduced lung microbial diversity, with *M. hyopneumoniae* predominance correlating with severe lung lesions. Nasal turbinates showed more microbial diversity but poorer detection compared to bronchoalveolar lavage fluid, emphasizing the importance of bronchoalveolar lavage fluid for microbiome analysis of the lower respiratory tract. Chapter 4 indicated that microchips are an effective alternative to monitor body temperature under experimental conditions, correlating well with rectal temperature. Movement analysis suggested that infected pigs move less, potentially serving as an early disease indicator. Lastly, parameters like percentage of air in the lung and average weight gain lacked utility in short-term *M. hyopneumoniae* trials.

Therefore, this thesis highlights the importance of understanding the microbiome, especially in relation to *M. hyopneumoniae*. In addition, the reduced diversity in the lung of infected pigs shows the impact of *M. hyopneumoniae* on respiratory health. Microchip-based temperature monitoring and movement analysis might offer promising avenues for early disease detection, including *M. hyopneumoniae*-related diseases.

Keywords: *Mycoplasma hyopneumoniae*, microbiome, respiratory tract, movement, thermochips, research, and swine.

Resumo – Explorando o microbioma respiratório e novos parâmetros de diagnóstico em suínos infectados com *Mycoplasma hyopneumoniae*

A demanda global por carne suína está aumentando devido ao crescimento populacional, o que exige a expansão do setor suinícola. No entanto, as doenças respiratórias, causadas por patógenos como o vírus PRRS, PCV-2 e *Mycoplasma hyopneumoniae*, apresentam desafios significativos na criação de suínos. Embora os avanços nas tecnologias de diagnóstico, como PCR e sequenciamento, tenham melhorado nossa compreensão do microbioma respiratório dos suínos, a interação entre o microbioma pulmonar e o *Mycoplasma hyopneumoniae* é menos clara. Sensores capazes de avaliar o comportamento, como o movimento, estão sendo introduzidos no setor de suínos. Eles podem fornecer informações adicionais sobre o bem-estar e até mesmo servir como indicadores precoces de doenças.

Dessa forma, a tese teve como objetivo investigar o microbioma respiratório de suínos infectados e não infectados por *M. hyopneumoniae*. Também teve como objetivo explorar novas ferramentas para apoiar o diagnóstico de *M. hyopneumoniae*. Os objetivos específicos incluiram a comparação da comunidade bacteriana no trato respiratório superior e inferior de suínos infectados e não infectados por *M. hyopneumoniae* e a avaliação do impacto da infecção no movimento e na temperatura corporal, usando câmeras e microchips respectivamente. Além disso, foram examinadas as associações entre movimento, temperatura e parâmetros de diagnóstico estabelecidos, como tosse, lesões pulmonares e carga bacteriana.

O Capítulo 3 mostra que os suínos infectados apresentaram uma diversidade microbiana pulmonar reduzida, com predominância de *M. hyopneumoniae* correlacionada com lesões pulmonares graves. Os cornetos nasais apresentaram maior diversidade, mas pior detecção em comparação com o fluido de lavagem broncoalveolar, enfatizando a importância do fluido de lavagem broncoalveolar para a análise do microbioma do trato respiratório inferior. O Capítulo 4 indicou que os microchips são uma alternativa eficaz para monitorar a temperatura corporal em condições experimentais, com boa correlação com a temperatura retal. A análise do movimento sugeriu que os

suínos infectados se movimentam menos, o que pode servir como um indicador precoce da doença. Por fim, parâmetros como a porcentagem de ar no pulmão e o ganho médio de peso não foram útjeis em experimentos de curto prazo com *M. hyopneumoniae*.

Portanto, esta tese destaca a importância de compreender o microbioma, especialmente em relação à *M. hyopneumoniae*. Além disso, a diversidade reduzida no pulmão de suínos infectados mostra o impacto de *M. hyopneumoniae* na saúde respiratória. O monitoramento de temperatura baseado em microchip e a análise de movimento podem oferecer caminhos promissores para a detecção precoce de doenças, inclusive doenças relacionadas *à M. hyopneumoniae*.

Palavras-chave: *Mycoplasma hyopneumoniae*, microbioma, trato respiratório, movimento, termochips, estudo experimental e suínos.

Samenvatting – Verkenning van het respiratoir microbioom en nieuwe diagnostische parameters bij varkens geïnfecteerd met *Mycoplasma hyopneumoniae*

De wereldwijde vraag naar varkensvlees stijgt door de bevolkingsgroei, waardoor uitbreiding van de varkensindustrie noodzakelijk is. Aandoeningen van de luchtwegen, veroorzaakt door pathogenen zoals het PRRS-virus, PCV-2 en *Mycoplasma hyopneumoniae*, vormen echter aanzienlijke uitdagingen in de varkenshouderij. Terwijl vooruitgang in diagnostische technologieën zoals PCR en sequencing ons inzicht in het respiratoire microbioom bij varkens heeft verbeterd, is de interactie tussen het longmicrobioom en *Mycoplasma hyopneumoniae* minder duidelijk. Sensoren die gedrag zoals beweging kunnen evalueren worden geïntroduceerd in de varkensindustrie. Deze zouden aanvullende informatie kunnen geven over welzijn en zouden zelfs kunnen dienen als vroege voorspellers van ziekte.

Daarom was het doel van dit proefschrift om het respiratoir microbioom van *met M. hyopneumoniae*-geïnfecteerde en niet geïnfecteerde varkens te onderzoeken. Het doel was ook om nieuwe hulpmiddelen te onderzoeken om de diagnose van *M. hyopneumoniae* te ondersteunen. Specifieke doelstellingen waren onder andere het vergelijken van de samenstelling van de kiemen in de bovenste en onderste luchtwegen van geïnfecteerde en niet-geïnfecteerde varkens en het beoordelen van de invloed van *M. hyopneumoniae* op de beweging en lichaamstemperatuur van biggen met behulp van cameramonitoren en thermische microchips. Daarnaast werden associaties tussen beweging, temperatuur en gevestigde diagnostische parameters zoals de ergheid van de hoest, longlaesies en bacteriële belasting onderzocht.

Hoofdstuk 3 laat zien dat geïnfecteerde varkens een verminderde microbiële diversiteit in de longen hadden, waarbij *M. hyopneumoniae* overheerste en correleerde met ernstige longlaesies. Neusschelpen vertoonden meer diversiteit maar een slechtere detectie in vergelijking met bronchoalveolaire lavagevloeistof, wat het belang benadrukt van bronchoalveolaire lavagevloeistof voor microbioomanalyse van de lagere luchtwegen. Hoofdstuk 4 gaf aan dat microchips een effectief alternatief zijn om de

lichaamstemperatuur onder experimentele omstandigheden te monitoren en goed correleren met de rectale temperatuur. Bewegingsanalyse suggereerde dat besmette varkens minder bewegen, wat mogelijk kan dienen als een vroege ziekte-indicator. Tot slot waren parameters zoals het percentage lucht in de longen en de gemiddelde gewichtstoename niet bruikbaar in kortdurende *M. hyopneumoniae*-proeven.

Daarom benadrukt dit proefschrift het belang van het begrijpen van het microbioom, vooral in relatie tot *M. hyopneumoniae*. Daarnaast toont de verminderde diversiteit in de longen van geïnfecteerde varkens de impact van *M. hyopneumoniae* op de gezondheid van de luchtwegen. Op microchips gebaseerde temperatuurmonitoring en bewegingsanalyse kunnen veelbelovende mogelijkheden bieden voor vroegtijdige opsporing van ziekten, waaronder *M. hyopneumoniae-gerelateerde* ziekten.

Trefwoorden: *Mycoplasma hyopneumoniae*, microbioom, luchtwegen, beweging, thermochips, onderzoek en varkens.

Chapter 1 | General Introduction

Pork is the second most consumed protein in the world, according to the latest data by the Food and Agriculture Organization, and its production is expected to grow by over 7,000 kilotons from 2023 to 2032, exceeding 129,377 kilotons by 2032 (OECD et al., 2023). Yet, considering the world's growing population and the increasing demand from consumers, the need for more animal protein in the next decades is evident. Thus, as a consequence, the pig industry is constantly growing and is also aiming for more profitability and sustainability. For a few years, pork consumers have been more aware of animal production in general and are looking for meat products originating from more sustainable production systems, which include improving animal health and welfare and production parameters, a lower usage of antimicrobials, among others.

When it comes to pig production, the European Union (EU) and Brazil are both important players in this market. The EU, which comprises 27 countries, holds the second-largest pig producer position, and is the biggest exporter of pork products in the world (USDA, 2024). Brazil is considered the fourth biggest producer of pigs and exporter of pork in the world (ABPA, 2023; USDA, 2024). Since it is free of major costly diseases like Porcine Reproductive and Respiratory Syndrome (PRRS) and African Swine Fever, it will continue to play a great role in the pig industry in the upcoming years.

Although animal health has overall improved in recent years, respiratory diseases in pigs remain a major challenge and a great cause of economic losses. Respiratory diseases are mostly multifactorial and involve several factors including infectious agents, poor management practices, and sub-optimal environment conditions. In addition, risk factors such as ammonia and dust concentration, and poor ventilation also play a great role in predisposing or favoring the development of respiratory diseases in pigs. Furthermore, infectious pathogens, either primary or secondary, should always be considered, especially in intensive pig farming, where the management and environmental conditions might be compromised due to the intensification of production. The most important respiratory pathogens, PRRS virus (PRRSV), Porcine circovirus 2 (PCV-2), and *Mycoplasma hyopneumoniae (M. hyopneumoniae)*, are primary pathogens and are also involved in the Porcine Respiratory Disease Complex (PRDC). This multifactorial syndrome is characterized by respiratory clinical signs, mainly pneumonia,

caused by the interaction of infectious viral and bacterial pathogens, as well as environmental stressors, management practices, and genetic factors (Eddicks et al., 2021; Martelli and Segalés, 2021).

When considering porcine respiratory diseases caused by bacterial agents, *M. hyopneumoniae* is the most important pathogen in swine production worldwide. It is present in the major pig-producing countries, with prevalence rates reaching over 70%, and is considered one of the main pathogens involved in the PRDC (Pieters et al., 2021; Pieters and Maes, 2019). It is a primary pathogen, so it can induce disease alone, but its interaction with other pathogens is known to worsen the disease. The interaction between primary pathogens like *M. hyopneumoniae* and opportunistic or secondary pathogens is yet not fully understood, especially in the lung, where sampling might be difficult, especially in living animals. In the past years, diagnostics of pathogens mostly depended on isolation, which can be time-consuming, labor-intensive, and limited to less fastidious agents. Thus, it was difficult to identify the microorganisms (pathogenic or not) present in the respiratory tract and how their interaction influenced the development of diseases. However, with the development of new technologies, such as polymerase chain reaction (PCR) and sequencing, investigating the respiratory microbiome becomes possible and will likely develop further. This will allow a better understanding of how the microbiome is established, the microorganisms that could play a beneficial role, the influence of pathogens like *M. hyopneumoniae* on the microbiome, and the interactions between microorganisms.

1.1. Respiratory tract of pigs

The respiratory tract represents the largest epithelial surface exposed to the outside environment in mammals, and pigs can breathe up to 12,000 liters of air every day (Yaeger and Alstine, 2019). Besides being a source of oxygen, the air might contain harmful gases (ammonia), particles (dust), and microorganisms (pathogenic or commensal), making it necessary for the development of a robust defense system. These defense mechanisms include the mucociliary apparatus, where mucus is produced, coating the epithelial surface and allowing particles to be trapped, and the immune system

that comprises innate and adaptive immune responses (Yaeger and Alstine, 2019). However, when this defense line is compromised, respiratory diseases might appear, often resulting in pneumonia, which can be linked to substantial losses in the swine industry (Ferraz et al., 2020; Pieters and Maes, 2019).

The respiratory tract can be divided into the upper and lower parts (Pirolo et al., 2023; Yaeger and Alstine, 2019). Pirolo et al. (2023) consider the nose, pharynx, and larynx as the upper respiratory tract (URT), and the trachea, bronchia, and lungs as part of the lower respiratory tract (LRT). The lung is divided into the right and left sides, comprising seven lung lobes in total. The right lung lobes are named cranial, cardiac or middle, diaphragmatic or caudal, and accessory, while the left lung is composed of the cranial, cardiac or middle, and diaphragmatic or caudal lobes (Figure 1) (Yaeger and Alstine, 2019). The URT's main functions include warming, humidifying, cleaning, and conducting the incoming air to the lower respiratory tract, as well as identifying pathogens and mounting an adaptive immune response (Yaeger and Alstine, 2019). In the LRT, the air is then delivered to the alveoli, where gas exchange happens (Yaeger and Alstine, 2019).

The respiratory tract of pigs, composed of the upper and lower parts, harbors a great diversity of microorganisms, which might be pathogenic or not. These microorganisms can interact with each other, favoring the development of respiratory diseases (Niederwerder, 2017; Pirolo et al., 2021). Thus, nowadays, with the development of sequence-based technologies, there is the opportunity to further investigate the microbial composition of the URT and LRT in diseased and healthy animals and how this composition is linked to the severity of diseases.

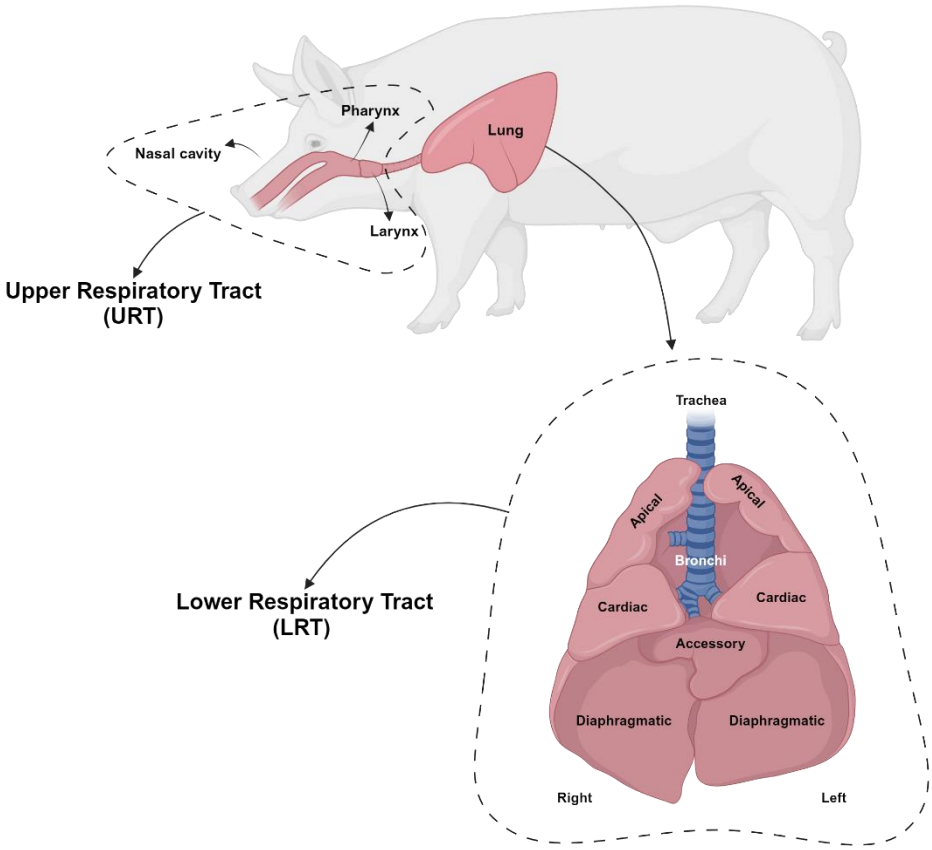

Figure 1. Simple description of the porcine respiratory system showing the division into upper and lower parts (created with BioRender).

1.2. The respiratory microbiome

Lungs were originally thought to be sterile because of some false beliefs and incorrect data interpretations (Wypych et al., 2019). For example, the human lung was considered sterile because of the inability to grow microbes from air samples (Dickson et al., 2016), and when DNA from microbes was detected, it was considered contamination (Wypych et al., 2019). However, the developments in culture-independent molecular techniques during the last decade have caused an important shift in knowledge. It was demonstrated that various microorganisms, such as bacteria, fungi, and viruses, collectively referred to as the microbiome, inhabit the lungs of both healthy and diseased individuals (Dickson et al., 2016; Faner et al., 2017; Hilty et al., 2010). Yet, as suggested by Hilty et al. (2010), it would be extraordinary if the LRT was able to maintain sterility in

the presence of high-volume airflow and the open communication with the oropharynx, in the URT. Thus, since the respiratory tract is constantly exposed to germs from inhaled air and also from microfluids from the oral cavity (Bassis et al., 2015), diverse communities of microorganisms can be identified (Dickson et al., 2016; Huttenhower et al., 2012).

In 1988, Whipps et al. (1988) defined a microbiome as a specific microbial community in a defined habitat with unique physio-chemical properties, encompassing not just the microorganisms present such as bacteria, archaea, viruses, fungi, algae, and small protists, but also their dynamic activities, comprising structural elements, genomes, metabolites, signal molecules, and surrounding environmental conditions (Marchesi and Ravel, 2015). However, when referring only to the microbes living in a defined microhabitat, the term microbiota has been suggested (Marchesi and Ravel, 2015). A recent study re-visited the definitions related to microbiome (Berg et al., 2020), suggesting the use of the term microbiome as mentioned above. However, to maintain consistency with the published information (Chapter 3), we refer to the term microbiome throughout this manuscript.

Although studies on the respiratory microbiome have increased exponentially in the last few years, little is known about its development and relationship with respiratory diseases in pigs, and how the longitudinal development of the respiratory microbiome occurs. It is also not clear which and where certain bacteria start the colonization of the respiratory tract, but it becomes more intense after birth (Pirolo et al., 2021). The diversity of microorganisms present in the URT and LRT seems to be directly related to the respiratory health of the animals, as a higher diversity is linked to healthy animals and a lower diversity is linked to diseased animals (Li et al., 2021; Pirolo et al., 2021; Rampelotto et al., 2022; Wang et al., 2018).

The microbiome composition can be influenced by several factors, including diet, genetics, age, environment, antibiotic exposure, management, and infection by pathogens (Niederwerder, 2017). Due to its immediate interface with the environment, the URT is the natural niche for respiratory bacterial and viral pathogens and the origin of consecutive respiratory tract infections (Bosch et al., 2016). In humans, it has been described that the nasal microbiota is established during infancy and changes with age (Bomar et al., 2018).

In pigs, a study showed a noticeable difference in the pre-weaning and post-weaning nasal microbiota. During early life, an apparent co-dominance between Proteobacteria and Firmicutes was observed, which shifted in dominance towards Proteobacteria after weaning (Slifierz et al., 2015) and also in slaughter-age pigs (Weese et al., 2014). Yet, it has been suggested that the nasal microbiota reaches stability or convergence towards stability approximately 2-3 weeks post-weaning (Slifierz et al., 2015).

It has been suggested that the LRT microbiome is greatly influenced by the URT, with a direct impact on the production, maintenance, and changes in the pulmonary microbial community (Li et al., 2024). In humans, it has been demonstrated that the nasal microbiome contributed, but not significantly to the composition of the lung microbiome in healthy individuals (Bassis et al., 2015). Yet, in the previous study, the researchers showed that the bacterial communities of the healthy lung overlapped with those found in the mouth but were found at lower concentrations (Bassis et al., 2015). Thus, there is strong evidence that the lung microbiome originates from the URT, although it is always in a transient mobile state (Bassis et al., 2015; Dong et al., 2022). In addition, when mouth breathing, saliva in the oral micro ecosystem could enter the respiratory tract, and certain behaviors like coughing could make the mucus of the respiratory tract and other substances enter the mouth, thereby achieving a mutual exchange between the oral cavity and the respiratory tract (Dong et al., 2022).

1.2.1. The microbiome composition in the upper and lower respiratory tract of pigs

As previously mentioned, the microbial composition of the respiratory tract can vary depending on the region (URT or LRT), and also within the region. Although the microbiome in the respiratory tract of pigs has been studied, most studies focused on the URT (Pirolo et al., 2021). When looking at recent literature, the most predominant genera found in the nasal cavity of healthy pigs are *Moraxella, Streptococcus, Clostridium* and *Lactobacillus* (Correa-Fiz et al., 2016; Espinosa-Gongora et al., 2016; Zeineldin et al., 2018), while *Actinobacillus, Pasteurella,* and *Glaesserella* (*Haemophilus)* formed the tonsillar core microbiome (Lowe et al., 2011). Wang et al. (2018) reported that the oropharyngeal microbial community of healthy piglets was closely related to that of the

tonsils, with *Streptococcus, Actinobacillus and Lactobacillus* forming the core microbiome. Pirolo et al. (2021) summarized the main phyla in the upper and lower respiratory tract of healthy pigs, as well as the factors affecting the abundance and bacterial diversity (Figure 2).

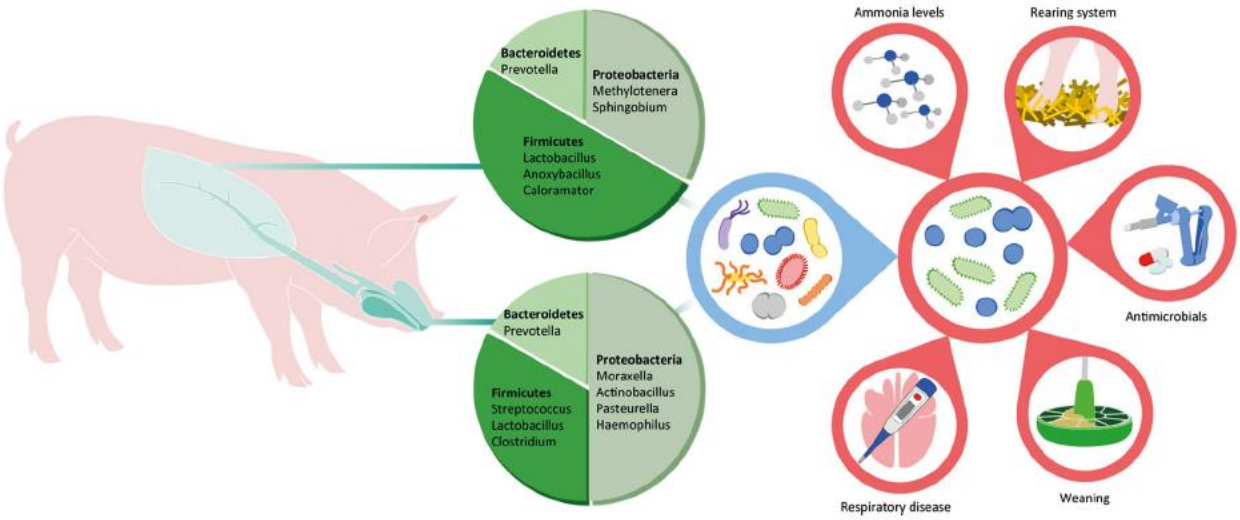

Figure 2. Bacterial topography of the upper and lower respiratory tract (pie charts) and factors shaping the porcine respiratory microbiome (Pirolo et al., 2021).

A recent study by Li et al. (2023b) reported *Firmicutes, Actinobacteria, Proteobacteria,* and *Bacteroidetes* as the dominant phyla in the nasal cavities of neonatal piglets. In addition, from the period of weaning through to the finishing stage, the proportion of *Proteobacteria* stayed consistent, while the phyla *Firmicutes, Bacteroidetes*, and *Actinobacteria* displayed fluctuations in response to the pigs' development. In finishing pigs, the core nasal microbiota comprised *Proteobacteria, Bacteroidetes, and Firmicutes* (Li et al., 2023b). Similar to the study of Wang et al. (2018), Rampelotto et al. (2022) observed that *Actinobacillus, Streptococcus Porphyromonas, Veillonella*, and an unclassified genus of *Pasteurellaceae* were more abundant in pigs with clinical signs of respiratory disease than in healthy pigs. Interestingly, the study also showed a high presence of *Mycoplasma* sp. in both groups, with and without clinical signs, but with low values in the nursery and finishing phases (Rampelotto et al., 2022). The composition of

the URT bacterial microbiota seems to differ significantly in pigs with or without respiratory clinical signs after weaning, and these differences might be maintained in the nursery phase (Rampelotto et al., 2022). Lastly, when comparing pigs with or without respiratory clinical signs, the composition of the URT bacterial microbiota differs significantly (Rampelotto et al., 2022).

It is known that bacterial diversity and abundance are lower in the LRT than in the URT (Pirolo et al., 2023). Pirolo et al. (2023) reported that lung microbiota was more similar to the tracheal and bronchial microbiotas than the microbiota in the URT. Few studies have investigated the LRT microbiome in healthy pigs, and the results are diverse. A recent study reported the genus *Glasserella, Streptococcus, Clostridium sensu stricto* 1*, Escherichia−Shigella,* and *Mycoplasma* as the most abundant microorganisms in the LRT of healthy pigs (Pirolo et al., 2023). In addition, other studies have reported *Methylotenera, Prevotella, Sphingobium, Lactobacillus, Escherichia, Mycoplasma, Lactococcus,* and *Macrococcus* as the predominant genera in healthy pigs (Huang et al., 2019; Li et al., 2021). A study by Li et al. (2021) with finishing pigs reported *Escherichia-Shigella*, *Mycoplasma*, *Lactococcus*, *Macrococcus*, and *Enterococcus* as the five most predominant genera in lungs of healthy animals. In addition, a study in Brazil, reported that the most common microbial families identified in healthy lungs were *Mycoplasmataceae*, *Bradyrhizobiaceae*, and *Flavobacteriaceae (Siqueira et al., 2017)*. Differently, Li et al. (2021) reported *Enterobacteriaceae*, *Mycoplasmataceae*, and *Streptococcaceae* as the most common bacterial families in healthy lungs. According to a recent meta-analysis on the URT and LRT microbiomes in domestic animals, Zeneldin et al. (2023) reported distinct differences in the microbial communities of the upper and lower respiratory tracts. In contrast, Li et al. (2023a) reported *Methylophilus* and *Variovorax* as the dominating genera in the lung of healthy pigs. Lastly, Pirolo et al. (2023) reported *Glasserella, Streptococcus, Clostridium sensu stricto* 1*, Escherichia−Shigella* and *Mycoplasma* as the most abundant genera in weaned pigs.

When looking at the microbiome data of the LRT from animals with respiratory disease, Siqueira et al. (2017) reported *Mycoplasmataceae*, *Flavobacteriaceae,* and *Pasteurellaceae* as the most common families. Another study reported *Aeromonas,*

Psychrobacter, *Acinetobacter*, *Vagococcus*, and *Streptococcus* as the most common genera in pigs with respiratory disease (Li et al., 2021). Yet, the authors also observed that the relative abundances of *Lactococcus*, *Enterococcus,* and *Staphylococcus* were much higher in the healthy group, while *Streptococcus* and *Glaesserella* (*Haemophilus)* were much higher in the diseased group (Li et al., 2021). In addition, Li et al. (2023a) reported *Mycoplasma*, *Methylophilus*, and *Ureaplasma* as the most abundant genera in pigs with lung lesions, and a higher relative abundance for *M. hyopneumoniae*, *M. hyorhinis*, and *Ureaplasma diversum* was reported in lungs with lesions than lungs without lesions. Lastly, it has been reported that the diversity of lung microbial community between healthy and diseased animals is significantly different, suggesting that the lung microbiotas were possibly associated with lung lesions (Li et al., 2023a; Li et al., 2021; Siqueira et al., 2017). A summary of the most relevant studies is shown below.

Table 1: Summary of the main microbiome studies of the respiratory tract in pigs from 2011 to 2020. Adapted from Pirolo et al. (2021)

Authors	Year	Number of Animals	Age (weeks)	Sample	Controls	Main Finding
Slifierz et al.	2015	10	0-7	Nasal swab	Absent	The nasal microbiota of pigs undergoes a remarkable evolution during the first 7 weeks of life
Pena Cortes et al.	2018	34	0-4	Tonsil swab	Extraction control	The tonsillar microbiome of piglets does not show composition stability until 3 weeks, and shared similarities with the sow vaginal and teat skin microbiome
Pena Cortes et al.	2018	16	0-19	Tonsil swab	Extraction control	Significant community shifts during the development of the tonsillar microbiome are correlated with disruption events, including weaning, changes in feed, antibiotic treatment, and movement to new housing
Lowe et al.	2012	12	18-20	Tonsil swab and tissue	Absent	*Actinobacillus, Pasteurella* and *Haemophilus* form the tonsillar core microbiome
Espinosa-Gongora et al.	2016	100	Last 3 weeks of production	Nasal swab	Absent	The nasal microbiota may play a role in the individual predisposition to *Staphylococcus aureus* nasal carriage in pigs
Correa-Fiz et al.	2016	100	3-4	Nasal swab	Absent	The composition of the nasal microbiota of healthy piglets was uncovered and different phylotypes were shown to be significantly altered in animals depending on the clinical status of the farm.
Weese et al.	2014	20	1–2 weeks prior to slaughter	Nasal swab	Absent	The nasal cavity of slaughter-age pigs harbours a rich and diverse microbial community that can be influenced by diet and farm management practices
Siqueira et al.	2017	40	Not specified	BAL	Absent	In farms with a history of chronic respiratory problems, *Mycoplasma hyopneumoniae* is present in lungs with or without enzootic pneumonia signs
Li et al.	2020	28	38	BALF	Absent	Bacterial taxonomic communities of healthy and diseased swine differed at the genus level
Chrun et al.	2021	24	5-7	Nasal swab	Absent	PRRSV-2 and H3N2 alone or in combination differentially altered the abundance and distribution of bacterial families
Blanco-Fuertes et al.	2021	74	3-4	Nasal swab	Absent	Weaned piglets with polyserositis caused by *M. hyorhinis* or *G. parasuis* present differences in microbiota composition
Almeida et al.	2022	12	4-8	Nasal turbinate /BALF	Absent	Colonization by M. hyopneumoniae, this pathogen can alter the number of bacterial species in the lungs

Li et al.	2022	10	Slaughtered pigs	BALF	Absent	Differences in the diversity of lung microbial community across the lung-health and lung-lesion groups were observed
Rampelotto et al.	2022	20	3-24	Nasal swab	Absent	Composition of the upper respiratory tract bacterial microbiota differs significantly when comparing pigs with or without respiratory clinical signs after weaning these differences were not observed at the finishing phase
Nielsen et al.	2023	20	3-9	Nasal wash/swab	Extraction control	B. bronchiseptica colonization can disturb the upper respiratory tract microbiota

1.2.2. Respiratory diseases and the microbiome

In pigs, microbiome diversity and composition in the respiratory tract likely play an important role in regulating host immune homeostasis and preventing PRDC (Niederwerder, 2017). Pirolo et al. (2023) suggested that the relationship between URT and LRT microbiomes could explain differences in PRDC progression and severity, as the URT serves as a primary reservoir for pathogens that might reach the lung and cause disease. The study also reported that the microbiota in the choana was similar in community composition to the tracheal microbiota, suggesting that the posterior portion of the pig nasal cavity is the primary source of bacteria for the LRT (Pirolo et al., 2023).

It has been demonstrated that *M. hyopneumoniae* infection affects the respiratory tract of pigs, and it seems to facilitate the proliferation of other bacteria, especially those related to PRDC and secondary pathogens (Almeida et al., 2022; Surendran Nair et al., 2019; Valeris-Chacin et al., 2021). The bacterium tends to influence the LRT more than the URT (Almeida et al., 2022; Sonalio et al., 2022), which is likely due to its tropism for the LRT (Marois et al., 2007; Pieters et al., 2021; Pieters and Maes, 2019). Under experimental conditions, Almeida et al. (2022) observed that *M. hyopneumoniae* infection had only a minor influence on the nasal microbiota diversity, while it had a great impact on the LRT. Wang et al. (2018) observed the genera *Moraxella, Veillonella,* and *Porphyromonas* as the most prevalent bacteria in pigs with respiratory disease (cough, fever, joint swelling, and wheezes) under field conditions, suggesting that they may play a potential role in the development of the disease. Another study reported that when animals developed clinical signs of Glasser's disease, the abundance of *Mycoplasma* increased in those animals (Correa-Fiz et al., 2016). Furthermore, a study by Correa-Fiz et al. (2019) suggested that not using perinatal antimicrobials resulted in an increase in nasal microbiota diversity, which was accompanied by a general health improvement in the piglets. Lastly, a lower relative abundance of several potentially pathogenic bacteria, such as *Mycoplasma, Glaesserella* (*Haemophilus)*, *Campylobacter* and *Treponema*, was observed in the nasal microbiota of healthier animals (Correa-Fiz et al., 2019).

Although some studies have investigated the composition and diversity of the respiratory tract in healthy and diseased pigs, little information is known about the

influence of specific pathogens such as *M. hyopneumoniae* on the respiratory microbiome under field conditions.

1.2.3. Sequence-based technologies for microbiome analysis

Approximately 100 years ago, when the concept of sterility first emerged, it was difficult to analyze the microbial composition of the airways, and detection was solely based on culture methods that, as we now know, are only capable of identifying a small percentage of the bacteria present in complex samples (Hilty et al., 2010; Staley and Konopka, 1985). However, with the development of other detection methods such as PCR and next-generation sequencing (NGS), the study of the lung microbiome has gained more attention (Johnson et al., 2019; Li et al., 2024), allowing many new studies to be performed.

Different sequencing methods were developed during the last decades, including first (e.g. Sanger), second (e.g. Illumina), and third generation (e.g. Nanopore) technologies. The first generation sequencing (Sanger sequencing) uses nucleotides (ddNTPs) tagged with specific fluorescent dyes, which allow the identification of the specific nucleotide according to its corresponding color (Ambardar et al., 2016). In the second generation method, many templates from the same sample are sequenced in parallel in a single run, producing a large amount of short-read data (Ambardar et al., 2016). Third generation sequencing results in a longer read length at competitive costs (Ambardar et al., 2016).

In recent years, the second and third generation sequencing methods have slowly replaced classical Sanger sequencing, especially for metagenomics and microbiome in general (Bharti and Grimm, 2019). Second generation sequencing includes different approaches, including the Illumina sequencing (Caporaso et al., 2012), which uses a sequencing-by-synthesis approach. Thus, as a conserved component found in all DNA-dependent species, the 16S rRNA gene is extremely useful for microbiome analysis using NGS. This gene is composed of nine highly variable areas (V1–V9), which allow the examination of differences between different bacteria (Campbell et al., 2022; Whiteside et al., 2021). This method, also known as short-read sequencing, is however limited when

considering species-level identification (Whiteside et al., 2021). It provides relatively short reads (~2× 300 bp) at a lower cost compared to other high-throughput sequencers (Bharti and Grimm, 2019). Even though Illumina platforms provide high accuracy data and are more cost-effective, they only provide limited read length, giving space to other technologies such as third generation sequencing (e.g. Nanopore), which results in longer reads of about 1.10^6 bp (Bharti and Grimm, 2019) at a competitive price. As RNA and DNA molecules have a great size, sequencing them in short amplified fragments complicates the reconstruction and counting of the original molecules; therefore, long reads can be beneficial to improve de novo assembly, mapping certainty, and detection of structural variants (Amarasinghe et al., 2020). Furthermore, sequencing of DNA and RNA with long-read sequencers seem to eliminate amplification bias while preserving base modifications (Depledge et al., 2019).

1.2.4. What to consider in microbiome studies

Overall, the respiratory tract is the port of entry of several microorganisms that colonize different regions such as nasal cavity, tonsils, oropharynx, trachea, and lung. The most common samples to assess the microbiome of the URT include swabs (nasal, tonsil, oropharyngeal) or tissue (nasal turbinate or tonsils), while for the LRT, bronchoalveolar lavage fluid (BALF) is most commonly used (Pirolo et al., 2021). In addition, different sequencing tools and platforms are available, thus selecting the most suitable methods is important (Pirolo et al., 2021).

When looking at studies of the respiratory microbiome in pigs, over 76% of them evaluate the URT, which might be linked to the robustness and stability of the URT microbiome in comparison to the LRT (Li et al., 2024; Pirolo et al., 2021), but could also be related to the ease of sampling. Still, most studies investigating the porcine airway microbiome before 2021 did not include positive and negative control samples (Pirolo et al., 2021). In addition to that, DNA contamination is a major challenge, especially for samples with low microbial biomass such as BALF samples, especially in the case of *in vivo* sampling. The low amount of starting material may be effectively swamped by the contaminating DNA, which could originate from the URT and also from reagents (sterile,

but not DNA-free) (Salter et al., 2014; Whiteside et al., 2021). In 2014, Salter et al. (2014) reported that bacterial DNA contamination in extraction kits and laboratory reagents can significantly influence the results of microbiota studies, particularly when investigating samples containing a low microbial biomass. In addition, when sampling the LRT of live animals where the swab or catheter has to pass through the URT, contamination is likely to occur (Bassis et al., 2015; Carney et al., 2020). Consequently, BALF from the whole lung (post-mortem) was suggested as a robust and diverse sample, and with a minimal risk for contamination from the URT (Carney et al., 2020).

To decrease costs, pooling of samples can be considered (Almeida et al., 2022; Siqueira et al., 2017). A recent study showed that pooling of samples before DNA amplification to estimate community level diversity is a valuable measure to consider in population studies (Ray et al., 2019). To guarantee representative results and to avoid bias, the number of samples should be defined prior to the start of the study (Bharti and Grimm, 2019).

1.3. *Mycoplasma hyopneumoniae*-related disease

1.3.1. Epidemiology

With a worldwide distribution, *M. hyopneumoniae* is considered to affect up to 70% of the swine herds (Pieters and Maes, 2019), including the most important pig-producing countries (China, United States, and Brazil) (USDA, 2023). In addition to its high prevalence, significant economic losses, mainly linked to decreased performance and increased treatment and vaccination costs, are often associated with the infection (Boeters et al., 2023; Pieters and Maes, 2019). For instance, a study by Ferraz et al. (2020) concluded that animals without Enzootic Pneumonia (EP) lesions can be up to $ 6.55 more profitable than animals with lesions. This results from a decrease of 1.8 g in average daily gain for every percent of increase in area of lung consolidation. In addition, Donko et al. (2005) reported that pigs in the finishing phase suffering of pneumonia and atrophic rhinitis can have up to 11.5% reduction in the average daily gain. Lastly, several studies reported the negative impact of lung consolidation and pleurisy on the performance of pigs (Maes et al., 2023).

Although pigs of all ages are susceptible to *M. hyopneumoniae* infection, the disease is primarily observed in growing and finishing pigs and is characterized by its chronic condition, high morbidity, and low mortality (Pieters and Maes, 2019). Thus, once the infection is established in the herd, it tends to remain endemic (Pieters et al., 2021).

Mycoplasma hyopneumoniae transmission occurs by direct or indirect contact with contaminated respiratory secretions. Direct transmission by nose-to-nose contact between infected and susceptible animals is the most important one (Pieters et al., 2021). Indirect transmission can take place via aerosols over short or long distances (Cardona et al., 2005; Stark et al., 1998), even up to 9.2 km between farms (Otake et al., 2010). Apart from the route, climate conditions such as precipitation and temperature might also influence the efficacy of airborne transmission (Segales et al., 2012; Vangroenweghe et al., 2015). *Mycoplasma hyopneumoniae* survival in the environment is believed to be short, as the pathogen is susceptible to UV-light and dry environments (Jorsal and Thomsen, 1988). In addition, Browne et al. (2017) showed in an *in vitro* study that different *M. hyopneumoniae* strains were able to survive for eight days at 4°C in dust.

1.3.2. Etiology

Mycoplasma hyopneumoniae was proposed as a new species in 1965 by Maré and Switzer (1965), and has been recognized as a major respiratory pathogen in pigs for decades under that name (1965). However, in 2018, Gupta et al. (2018) proposed a taxonomic reclassification to *Mesomycoplasma hyopneumoniae*, which was recently validated by the International Committee on Systematics of Prokaryotes (ICSP) under the International Code of Nomenclature of Prokaryotes (ICNP) (Oren et al., 2023). Nevertheless, since *Mycoplasma hyopneumoniae* is mentioned as a basonym in the List of Prokaryotic names with Standing in Nomenclature (LPSN) (Parte et al., 2020) and the articles included in this PhD thesis used the “old” nomenclature, *Mycoplasma hyopneumoniae* (*M. hyopneumoniae*) was chosen to be consistently used throughout this thesis.

Mycoplasma hyopneumoniae belongs to the class *Mollicutes*, order *Mycoplasmoidales*, family *Metamycoplasmataceae*, and genus *Mesomycoplasma* (Gupta

et al., 2018). The bacteria included in this class lack a cell wall, require sterols to grow, and are aerobic or facultatively anaerobic (Gupta et al., 2018; Mare and Switzer, 1965). They have a small genome, ranging from 580 to 1,350 kb, a low G+C content, and are fastidious to grow *in vitro*, mainly due to the absence of some genes involved in biosynthetic pathways. Thus, it depends on the host for the supply of certain components, as is the case for fatty acids and cholesterol (Razin et al., 1998). Although a specific medium (Friis, 1975) was developed to grow *Mycoplasma* species, isolation of *M. hyopneumoniae* is not used as a common diagnostic method (Pieters and Maes, 2019), especially because of slow and fastidious growth. It takes a few weeks to obtain isolates, if successful at all. Within the abovementioned genus, three species of *Mycoplasma* (*M.*) in pigs are reported – namely *M. hyopneumoniae*, *M. hyorhinis*, and *M. flocculare*. However, other species such as *M. hyosynoviae*, *M. suis*, *M. parvum*, and *M. hyopharyngis* are also commonly found in pigs (Hoelzle, 2021; Rycroft, 2021; Sonalio et al., 2021), but according to the new classification proposed by Gupta et al. (2018), these species belong to other genera than *Mesomycoplasma*.

Up until now, 26 *M. hyopneumoniae* genomes have been sequenced and deposited into the NCBI database (Toledo et al., 2023), including the first *M. hyopneumoniae* isolate, the J strain (RF Goodwin and Whittlestone, 1963). With the increasing availability and affordability of whole-genome sequencing techniques, more information regarding genomic diversity and interaction between pathogens can be obtained (Jarocki and Djordjevic, 2021). Still, direct genomic comparisons tend to be difficult, as strain characterization tends to be done using different tools and methods (Betlach et al., 2019; Jarocki and Djordjevic, 2021). However, regardless of the method used, *M. hyopneumoniae* strains show a high genetic diversity, resulting in differences in virulence, as well as antigenic variation (Charlebois et al., 2014; Dos Santos et al., 2015; Madsen et al., 2007; Meyns et al., 2007; Michiels et al., 2017b; Vicca et al., 2003a). In this sense, Leal Zimmer et al. (2019) showed that *M. hyopneumoniae* strain 7448 secreted a higher number of adhesins and many hypothetical proteins *in vitro* when compared to the *M. hyopneumoniae* strain J, which was suggested as a potential virulence factor. Different adhesins, lipoproteins, and aminopeptidases have been reported as virulence factors

(Kuhnert and Jores, 2021; Leal Zimmer et al., 2020), and they might have important roles in the adhesion, cytotoxicity, and immunomodulation, respectively (Ferrarini et al., 2016; Kuhnert and Jores, 2021).

1.3.3. Pathogenesis

The ability of a microorganism to cause disease is known as pathogenicity, which is directly related to the presence of virulence factors in these microorganisms. When considering *M. hyopneumoniae* infections, a cascade of events needs to happen for successful colonization and establishment of the disease, making the process complex, and up till now, not fully understood (Kuhnert and Jores, 2021; Pieters and Maes, 2019).

First, for the pathogen to cause an infection, it must reach the ciliated epithelial cells lining the respiratory tract. However, this journey can be difficult due to obstacles like coughing, turbulent air flow, and mucus (Bustamante-Marin and Ostrowski, 2017; Gamage et al., 2018; Sjaastad, 2003). The respiratory cilia beat in metachronal waves (like a brush) to clear the airways of pathogens and inhaled particles stuck in the mucous layer that lines the respiratory tract, which will be moved towards the mouth, then being swallowed or expectorated (Bustamante-Marin and Ostrowski, 2017).

Second, if *M. hyopneumoniae* can reach the ciliated epithelium of the trachea, bronchi, and/or bronchioles, it needs to adhere to the cilia to establish infection. At least 35 proteins have been associated with *M. hyopneumoniae* adhesion (Leal Zimmer et al., 2020), but many more could be involved in the process. The most important adhesins of *M. hyopneumoniae* are P97 and P102, which are also the most often studied paralogous families (Kuhnert and Jores, 2021). When proteins have more than 30% amino acid identity over 70% of the length, they are defined as paralogous gene families (Minion et al., 2004). In addition, using a BLASTP analysis of the *M. hyopneumoniae* genome, Minion et al. (2004) revealed several paralogs of both the P97 and P102 genes.

Furthermore, the cleavage fragments of P97, P102, and paralogs, like P40, P42, P45, P48, P50, P60, P85 and P120, can bind cilia, heparin, fibronectin, and plasminogen (Bogema et al., 2012; Bogema et al., 2011; Kuhnert and Jores, 2021; Seymour et al., 2010; Seymour et al., 2012). In addition, P159, P216, and P68 have also been described

as adhesins belonging to the P97/P102 families (Kuhnert and Jores, 2021), and also associated with binding to the cilia. In addition, besides binding to the cilia, P68 was recently associated with apoptosis of peripheral blood mononuclear cells and alveolar macrophages (Liu et al., 2019). Moonlighting cytosolic proteins on the cell surface such as L-lactate dehydrogenase and the elongation factor Tu (Tacchi et al., 2016; Yu et al., 2018) were reported to bind fibrinogen, heparin, and actin, suggesting their role as adhesins. Furthermore, the production of toxic metabolites like H_2O_2 was suggested to be a virulence factor of *M. hyopneumoniae* (Ferrarini et al., 2016) and to contribute to the differences in virulence among strains (Kuhnert and Jores, 2021).

Finally, once attached to the ciliated epithelium of the conducting airways, *M. hyopneumoniae* progressively colonizes the area (especially the cranioventral lung lobes), damaging the cilia and compromising the mucociliary clearance system (Kuhnert and Jores, 2021; Pieters and Maes, 2019). In addition, the host immune response is modulated by the bacterium, facilitating its persistence, and favoring the development of pulmonary lesions (Figure 1). Deeney et al. (2019) suggested that *M. hyopneumoniae* evades uptake by porcine alveolar macrophages even after supplementation with convalescent serum, indicating a potential immune evasion mechanism. Likewise, Raymond et al. (2018) showed that *M. hyopneumoniae* can enter swine epithelial cells (PK-15) via clathrin- and caveolae-mediated endocytosis and is able to survive phagolysosomal fusion. However, since the previous study worked with kidney cells (PK-15) *in vitro*, and *M. hyopneumoniae* is a respiratory pathogen this information might be less relevant *in vivo* (Ferrarini et al., 2016; Pieters and Maes, 2019). Lastly, Paes et al. (2018) reported that anti-actin antibodies can significantly inhibit adhesion and colonization of PK-15 cells, indicating that extracellular actin is an important receptor for *M. hyopneumoniae* (Leal Zimmer et al., 2020), possibly playing a role in reinfection.

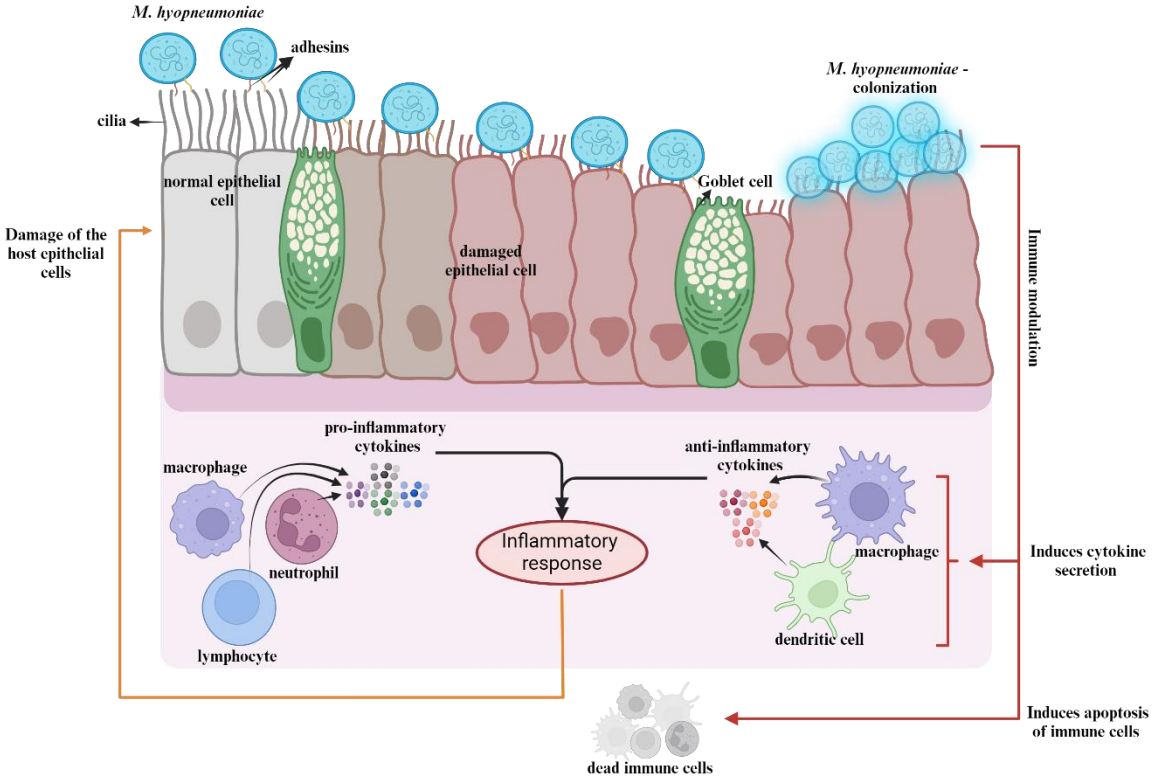

Figure 3. Schematic representation of *M. hyopneumoniae* adhesion to the respiratory cilia and immune response modulation. Adapted from Leal Zimmer et al. (2020).

The adhesion proteins bind to epithelial cells, causing signals of inflammation or invasion, also triggering the recruitment of immune cells into the lungs, such as macrophages and neutrophils (Leal Zimmer et al., 2020). In addition, virulence factors like lipoproteins and hemolysin C contribute to the cytotoxicity of *M. hyopneumoniae*, exacerbating tissue damage and inflammation while promoting the invasion of more immune cells (Leal Zimmer et al., 2019; Leal Zimmer et al., 2020)

The accumulation of mononuclear cells and infiltration of lymphocytes, plasma cells, and neutrophils in the alveolar lumina and septa upon *M. hyopneumoniae* infection are common histopathological alterations in the respiratory tract of pigs (Leal Zimmer et al., 2020). Rodríguez et al. (2016) confirmed the association between bronchiolar changes, including an increase in inflammatory cells and MUC5AC-producing goblet cells and broncho-alveolar lymphoid tissue hyperplasia with *M. hyopneumoniae* infection.

Recently, Almeida et al. (2020c) associated the expression of pro-inflammatory cytokines (IL-1α, IL-1β, IL-6, IL-8, TNF-α) and suppression of anti-inflammatory IL-10 with the development of macroscopic and microscopic lung lesions in *M. hyopneumoniae*-infected pigs. Therefore, these findings indicate that the host's immune response to infection might be a primary contributor to the development of cranioventral pulmonary consolidation lesions and pneumonia in pigs (Leal Zimmer et al., 2020).

1.3.3.1. Interaction with other pathogens

Mycoplasma hyopneumoniae is commonly found in combination with other pathogens such as *Actinobacillus pleuropneumoniae (A. pleuropneumoniae)*, *Pasteurella multocida* (*P. multocida)*, *M. hyorhinis*, *Glaesserella parasuis* (*G. parasuis)*, PCV-2, PRRSV, and swine Influenza A Virus (swIAV), which are also known to cause damage to the respiratory tract. Although the pathogenesis of these microorganisms is well-understood, little is known about the additive or synergetic effects of potential coinfections and the associated clinical signs (Marois-Créhan et al., 2021). *Actinobacillus pleuropneumoniae* induces a serofibrinous, and necrotizing pleuropneumonia and has been reported to persist in tonsillar crypts (Marois-Créhan et al., 2021). Marois et al. (2009) showed that the presence of *A. pleuropneumoniae* (serotype 9) may be clinically unnoticed and that *M. hyopneumoniae* may potentiate infections with this pathogen. Furthermore, this coinfection also results in immune response modulation, with *M. hyopneumoniae* decreasing the phagocytic capacity of macrophages, favoring tissue damage induced by *A. pleuropneumoniae* (Caruso and Ross, 1990). A suppurative bronchopneumonia is induced by *P. multocida* infection, a secondary pathogen capable of worsening clinical signs and lesions in *M. hyopneumoniae*-infected pigs (Amass et al., 1994a; Ciprián et al., 1988). Park et al. (2016) reported that *M. hyopneumoniae* enhances the adherence of *P. multocida* type A by increasing the L-fucosyl composition in bronchial and bronchiolar epithelial cells.

Mycoplasma hyorhinis is also listed as a contributing agent for the PRDC and has been associated with a decreased growth rate in pigs, although no associations were observed with the severity of pneumonia (Fourour et al., 2019). Likewise, Ferreira et al.

(2021b) could not determine associations between the combined infection with *M. hyorhinis* and *M. flocculare* with the degree of cranioventral pulmonary consolidation. Interestingly, the previous study also reported that *M. flocculare* load decreases when the *M. hyopneumoniae* load increases and is negatively associated with the severity of the lung lesions (Ferreira et al., 2021b). Up until now, *G. parasuis* infection in combination with *M. hyopneumoniae* has not been experimentally tested. In addition, its DNA is less often found in pig lungs, when compared to other pathogens, such as *M. hyopneumoniae* (Marois-Créhan et al., 2021).

When considering the viral respiratory pathogens, the interaction between *M. hyopneumoniae* and PRRSV has been reported, but the mechanisms of interaction remain to be understood (Thacker et al., 1999; VanAlstine et al., 1996). Thacker et al. (1999) observed more severe PRRSV-induced pneumonia in pigs previously infected with *M. hyopneumoniae*, while an initial PRRSV infection did not affect the severity of pneumonia induced by *M. hyopneumoniae*. In addition, increased levels of IL-10, IL-1α, IL-1β, IL-8, and TNFα may play a role in PRRSV and *M. hyopneumoniae* coinfection, exacerbating the severity and duration of pneumonia (Thanawongnuwech et al., 2004; Thanawongnuwech et al., 2001a; Thanawongnuwech et al., 2001b). Immunomodulation and lymphoid depletion are common characteristics associated with PCV-2 infections, making it one of the most important pathogens associated with the PRDC. In addition, it has been demonstrated that an *M. hyopneumoniae* infection increased the amount of PCV-2 copy numbers in sera, prolonged the presence of PCV2 antigen in lymphoid and lung tissues, and increased the amount of PCV-2 antigen in lung tissues in dual-infected pigs (Opriessnig et al., 2004). Furthermore, a recent study by Suh et al. (2024) reported that the potentiation effect of *M. hyopneumoniae* on PCV2d was observed only in pigs inoculated with *M. hyopneumoniae* followed by PCV-2d or simultaneously infected with both pathogens (PCV-2 and *M. hyopneumoniae*), thus, suggesting that the clinical outcome depends on the infection order.

Studies have investigated the interaction between swIAV and *M. hyopneumoniae,* indicating a more severe disease when swIAV infection occurred after *M. hyopneumoniae* infection (Marois-Créhan et al., 2021). In addition, higher inflammatory responses were

observed after swIAV infection in *M. hyopneumoniae*-infected pigs (Deblanc et al., 2016), with increased production of the pro-inflammatory cytokines IL-6, IL-1β, and TNF-α. Thus, the abovementioned studies suggest that *M. hyopneumoniae* is more likely to act as a primary pathogen and tends to induce more severe disease in coinfections.

1.3.4. Clinical signs

The clinical signs related to *M. hyopneumoniae* infections are directly dependent on the coinfections, and, as described by Carr et al. (2021), three major clinical-pathological conditions are observed.

When *M. hyopneumoniae* is the only pathogen affecting the pigs, the disease is known as Porcine Mycoplasmosis (Carr et al., 2021). This condition is mainly seen in experimentally inoculated pigs, where high doses of the bacterium are used (Almeida et al., 2020c; Beuckelaere et al., 2022; Matthijs et al., 2019a; Mechler-Dreibi et al., 2021b; Michiels et al., 2017a; Michiels et al., 2018; Vicca et al., 2003a). In this case, first signs of mild coughing are usually observed from seven days post infection (pi) onwards, reaching the maximum severity by four to five weeks pi (Almeida et al., 2020c; Beuckelaere et al., 2022; Matthijs et al., 2019a; Mechler-Dreibi et al., 2021b; Michiels et al., 2017a; Michiels et al., 2018; Vicca et al., 2003a). In case *M. hyopneumoniae* is introduced into a negative herd, coughing will start within a week after infection. Other clinical signs like pyrexia (fever), abortion, infertility, and dyspnea might be observed (Carr et al., 2021). In addition, morbidity can reach 100% in a couple of weeks, likely placing the herd into an endemic situation within half a year (Carr et al., 2021). However, since *M. hyopneumoniae* infection is mostly accompanied by coinfections, Porcine Mycoplasmosis is rarely seen in pig farms around the world (Carr et al., 2021).

Differently, EP, one of the most common conditions observed in the field, is characterized by *M. hyopneumoniae* infection associated with secondary pathogens (mostly bacteria), like *P. multocida*, *A. pleuropneumoniae*, *G. parasuis*, and *S. suis* (Carr et al., 2021; Sibila et al., 2009). Although EP can occur within all age groups, growing and finishing pigs tend to be the most frequently affected ones. Similar to Porcine Mycoplasmosis, EP is represented by a non-productive (dry) cough of different duration

and severity, mild fever, and decreased performance (Pieters and Maes, 2019). The clinical signs depend directly on the coinfecting pathogens, the virulence of the strains, the initial infectious dose, the health status of the herd, environmental conditions, etc. The onset of coughing is first observed in a few pigs, and because of the slow transmission rate, animals get infected at different times, allowing the pathogen to persist in the herd for a long time (Carr et al., 2021; Pieters and Maes, 2019). Besides (Opriessnig et al., 2011; Petri et al., 2023), *M. hyopneumoniae* and *P. multocida* concomitant infection results in elevated rectal temperatures, severe cough, and more extensive lung lesions (Amass et al., 1994b; Ciprián et al., 1988). Similarly, Marois et al. (2009) reported that pigs experimentally infected with *M. hyopneumoniae* at six weeks of age and with *A. pleuropneumoniae* (four weeks later) presented very severe clinical signs, which included hyperthermia, coughing, severe decrease in the average daily weight gain (ADWG), and death. Last, although being described as secondary pathogens in the PRDC and EP*, G. parasuis* and *S. suis* interaction with *M. hyopneumoniae* remains unclear. A recent study reported a high occurrence of these pathogens in lung samples, but no significant correlation was observed between these pathogens and lung lesions (Petri et al., 2023).

When viral pathogens are also involved – mostly PRRSV, PCV-2, and swIAV – the condition is referred to as PRDC, a multifactorial disease that does not necessarily need to be associated with *M. hyopneumoniae* infection (Carr et al., 2021; Maes et al., 2018c), although most farms worldwide are *M. hyopneumoniae*-positive. As the severity of the main clinical sign (dry cough) can be directly influenced by the coinfecting pathogens, different outcomes can be observed, especially under field conditions. Furthermore, fever, dyspnea, decreased feed efficiency, decreased feed intake, and decreased growth rate are often seen in clinical cases of PRDC (Opriessnig et al., 2011). As described by Thanawongnuwech et al. (2004), there is a synergy between PRRSV and *M. hyopneumoniae* when it comes to the severity and duration of the disease, which may be due to the inflammatory responses elicited in the course of the disease (Thanawongnuwech et al., 2001a). Regarding the coinfection by *M. hyopneumoniae* and PCV-2, Opriessnig et al. (2004) reported that the former potentiated the severity of PCV2-associated lung and lymphoid lesions and increased the incidence of respiratory disease.

On the other hand, potentiation of clinical signs in swIAV and *M. hyopneumoniae* infection were not observed by Thacker et al. (2001), Deblanc et al. (2016), or for one subtype of swIAV in the studies of Deblanc et al. (2016; 2012), suggesting that maybe the relationship between the two pathogens could be independent of each other, where each pathogen induces its normal course of pneumonia. Interestingly, in the study of Deblanc et al. (2012), one subtype of swIAV (avian-like influenza) caused a significant increase in the macroscopic lesion score when it was coinfected with *M. hyopneumoniae*.

However, since the most important respiratory diseases in pig production – namely PRDC and EP – involve the interaction between several pathogens, understanding the composition of the respiratory microbiome in the upper and lower respiratory tract of *M. hyopneumoniae*-infected and *M. hyopneumoniae*-free pigs may shed some light into this complex world of microbial interactions (Niederwerder, 2017; Siqueira et al., 2017).

1.3.5. Lesions

Evaluation of lung lesions is important for monitoring respiratory diseases around the world, especially those associated with *M. hyopneumoniae*, which is mostly done during the slaughter process, but also under experimental conditions. The lung lesions may be more extensive, exhibit mucopurulent discharge in the airways, a firmer texture, and a fluctuating greyish color in the lung tissue if additional bacterial infections are present (Maes et al., 2023). Thus, to get a better idea of the lesions associated with *M. hyopneumoniae* infections, further information is provided below.

1.3.5.1. Macroscopic lung lesions

M. hyopneumoniae infection and lesions are restricted to the respiratory tract, but the pathogen can be detected in other tissues (Marois et al., 2007). The macroscopic lesions are observed in the lung and normally present themselves as bilateral cranioventral pulmonary consolidation, also referred to as macroscopic lung consolidated lesion (MLCL). These lesions are mostly observed in the apical, cardiac, intermediate, and accessory lobes, and less often in the cranial portions of the diaphragmatic lobe (Carr et al., 2021; Pieters and Maes, 2019) (Figure 3).

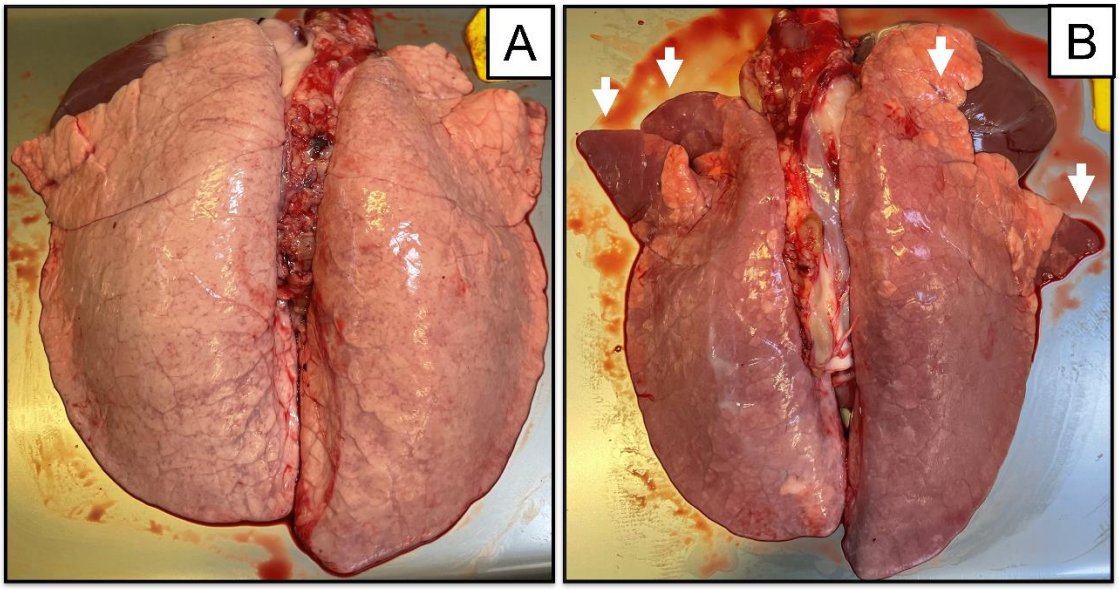

Figure 3. Porcine lungs with no lesions (A) and showing cranioventral consolidation (B), indicated by the white arrow.

Most lesions are seen in the right apical lobe due to the presence of the accessory bronchus, where *M. hyopneumoniae* first enters the lung when descending from the URT (Carr et al., 2021). At the beginning of the infection the affected area might be distinguished by its red-to-pink color, and later on, in a chronic condition, may look purple-to-grey (Carr et al., 2021; Pieters and Maes, 2019). Under experimental conditions, it was shown that the macroscopic lesions can appear between 1-2 weeks pi (Almeida et al., 2020c; Kobisch et al., 1993; Underdahl et al., 1980), reaching the maximum severity within 2-5 weeks pi (Carr et al., 2021; Kobisch et al., 1993; Pieters and Maes, 2019). Then, the lesions start to recover, resulting in fissures of collapsed alveoli, which are seen as tissue retraction and may be observed even after a couple of months pi (Kobisch et al., 1993), or possibly longer, when other pathogens are involved. Lastly, as mentioned by Maes et al. (2023), uncomplicated lesions show catarrhal-purulent exudate in the airways and the parenchyma with uniform color, while complicated lesions (by other infectious agents) are

larger in surface, have the presence of mucopurulent exudate in the airways, and an inconsistent greyish color of the parenchyma.

In PRDC cases, besides the cranioventral consolidation, animals might also show interstitial pneumonia with enlarged and non-collapsed lungs, with occasional interstitial edema and enlarged lymph nodes (Carr et al., 2021; Maes et al., 2023; Pieters and Maes, 2019). Opriessnig et al. (2004) reported that the total gross lung lesion scores were significantly more severe in pigs infected with *M. hyopneumoniae* and PCV-2. Likewise, Thanawongnuwech et al. (2004) and Thacker et al. (1999) showed that pigs infected with *M. hyopneumoniae* and PRRSV had a greater percentage of macroscopic lung lesions than pigs infected with only one of the pathogens. In dual infections with swIAV and *M. hyopneumoniae*, a significant increase in the macroscopic lesion score was reported (Deblanc et al., 2012; Thacker et al., 2001; Yazawa et al., 2004).

When considering coinfections of *M. hyopneumoniae* and *A. pleuropneumoniae*, Marois et al. (2009) reported that lung lesions were severe and corresponded to the pathogenicity of both pathogens combined; being more severe in pigs experimentally infected first with *M. hyopneumoniae* and then with *A. pleuropneumoniae*. Moreover, when *P. multocida* was associated with *M. hyopneumoniae*, more pneumonia lesions were observed in the lungs in comparison to animals with a single infection (Amass et al., 1994a). Similarly, Ciprián et al. (1988) reported more extensive exudative lung lesions in pigs infected with *M. hyopneumoniae* and *P. multocida* than in cases of single infection with either pathogen. A higher abundance of *M. hyopneumoniae* in lung samples with lesions suggestive of enzootic pneumonia was reported (Siqueira et al., 2017).

1.3.5.2. Microscopic lung lesions

Microscopic lesions related to *M. hyopneumoniae* develop earlier than the gross lesions, as the initial inflammatory response after infection starts shortly after the infection (Carr et al., 2021; Pieters and Maes, 2019). The development of the microscopic lesions can be divided into four stages (Pieters and Maes, 2019) and are characterized by:
I) accumulation of neutrophils in the lumen, bronchi, and alveoli, and few lymphocytes present; II) marked accumulation of neutrophils, fluid, and macrophages in alveoli;

proliferation of type II pneumocytes; accumulation of lymphocytes, histiocytes, and plasma cells around blood vessels and bronchi/bronchiole; development of peribronchial lymphoid nodules; III) peribronchial lymphoid hyperplasia, increased perivascular mononuclear cell accumulation, and development of alveolar pneumonia; IV) lots of lymphocytes and fewer plasma cells forming peribronchial cuffs, containing large lymphoid nodules that may compress bronchial lumens.

Overall, the microscopic lesions are characterized by hyperplasia of bronchoalveolar lymphoid tissue (Chae et al., 2021; Thacker et al., 1999). The presence of neutrophils is often seen when other bacterial pathogens are involved. When *M. hyopneumoniae* is associated with other bacterial pathogens such as *P. multocida*, exudative lesions were observed (Ciprián et al., 1988). Marois et al. (2009) did not reveal any differences between the lungs of pigs infected with *M. hyopneumoniae* alone or in association with *A. pleuropneumoniae*, where in both cases, infiltration of lymphocytes, interstitial pneumonia, lymphoid nodules, and collapse of the alveoli were observed.

Regarding the microscopic lesions observed in *M. hyopneumoniae* and viral pathogens (PRRSV, PCV-2, and swIAV), Thacker et al. (1999) showed that microscopic lesions consistent with *M. hyopneumoniae* infection were more severe in pigs infected with PRRSV and *M. hyopneumoniae*. Likewise, microscopic lesions were significantly higher in pigs challenged with *M. hyopneumoniae* and PRRSV than in the single-challenged groups (Opriessnig et al., 2004). Yet, lesions like peribronchiolar lymphoid hyperplasia (*M. hyopneumoniae*), interstitial pneumonia, peribronchial fibroplasia, and infiltration with macrophages and lymphocytes (PRRSV), were reported. Deblanc et al. (2012) reported that pigs infected with swIAV and *M. hyopneumoniae* showed exacerbation of bronchial pneumonia and superinfection with cellular exudates in the alveoli. Yazawa et al. (2004) reported more severe bronchial epithelial lesions (degeneration, hyperplasia, and formation of micro abscess) in double-challenged pigs (*M. hyopneumoniae* and swIAV).

In addition to evaluating the lesions microscopically, the percentage of lung area occupied by air can also be investigated (Beuckelaere et al., 2022; Matthijs et al., 2019a; Michiels et al., 2017a). This parameter is strongly associated with the severity of

microscopic lesions, being inversely proportional to the lymphohistiocytic infiltration in the lung tissue and the intrabronchiolar and bronchial exudate (Michiels et al., 2017a). However, since this evaluation is time-consuming and needs a large number of pictures and specific programs, it is mainly done for research purposes.

1.3.6. Diagnosis

Even though *M. hyopneumoniae*-related lesions and clinical signs are characteristic, they are not pathognomonic (Pieters and Maes, 2019). Consequently, it is necessary to rely on other methods for a precise diagnosis. Over the past years, with evolution of the technological field and optimization of some methods, like PCR, diagnosis of *M. hyopneumoniae* has been significantly enhanced, offering more reliable means of confirming the presence of the pathogen in swine populations. In addition, real-time PCR is vastly used in the field and research (Garcia-Morante et al., 2022), but diagnosis *of M. hyopneumoniae* tends to be different in field and research settings. Normally, in research settings, more parameters are analyzed such as isolation of the pathogen, microscopic lesions, macroscopic lesions with scoring, body temperature, among others. Thus, the next sections will describe the most common parameters used either in the field or research.

1.3.6.1. Clinical-pathological

The non-productive coughing is the most evident clinical sign of *M. hyopneumoniae* infection in pigs, but it can be triggered by several other factors (environment, other pathogens) (Chae et al., 2021). Although traditional methods, like observational scoring systems such as the ones proposed by Halbur et al. (1996) and Mores et al. (2001) to quantify coughing have proven valuable in indicating disease onset (Nathues et al., 2012; Pieters and Maes, 2019). Recently, sound-based monitoring technologies that can detect coughing like SoundTalks, have emerged (Berckmans et al., 2015; Ferrari et al., 2008). These will likely replace the traditional methods, mainly due to the ability to monitor pigs 24/7 and to detect small changes in sound intensity and frequency (Ferrari et al., 2008). However, to date, these technologies are not able to determine the etiology (Chae et al.,

2021; Nathues et al., 2012), thus, combination with other diagnostic methods is still necessary.

The presence and occurrence of cranioventral pulmonary consolidation lesions are often monitored during slaughter. This is useful to detect subclinical respiratory infections at the farm level, especially in pigs without obvious clinical signs but with lung lesions at slaughter (Chae et al., 2021; Maes et al., 2023). These lesions are also recorded under experimental conditions and can be evaluated with different, but well-correlated scoring systems (Garcia-Morante et al., 2016), as summarized by Maes et al. (2023) (Figure 4).

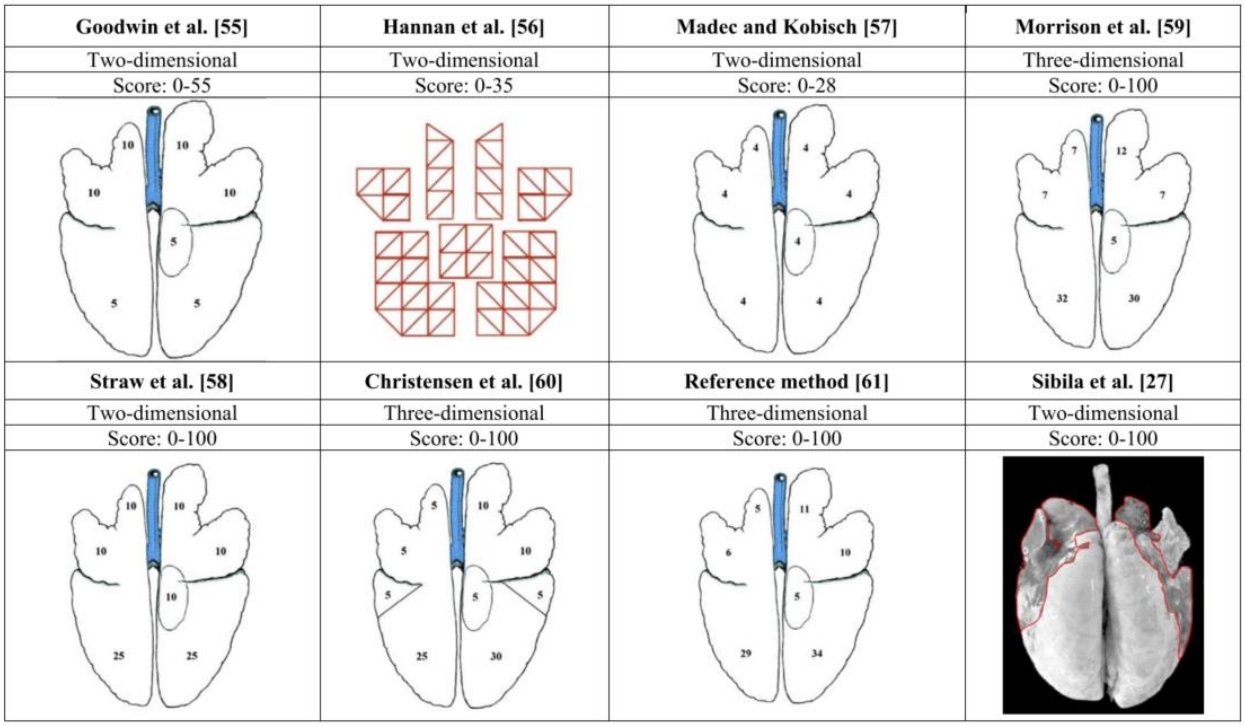

Figure 4. Methods commonly used to score macroscopic consolidated lesions in pigs (Garcia-Morante et al., 2016; Maes et al., 2023).

Among these methods is the scoring system developed by Hannah et al. (1982), which allows the investigator to carefully quantify lung lesions caused by *M. hyopneumoniae*. The evaluation consists of sketching the affected areas on a standard diagram of the pig lung. The score ranges from 0 (no lesions) to 35 points (entire lung

affected). Yet, the scoring of lungs can be adapted to digital applications to facilitate scoring and monitoring at the slaughterhouse (Ferraz et al., 2020; Przyborowska-Zhalniarovich et al., 2023). And such applications might also allow the combination of different scoring methods (Christensen et al., 1999; Madec F, 1982).

Following the evaluation of gross lesions, a histopathological analysis may be performed to differentiate between *M. hyopneumoniae*-induced lesions from lesions caused by other infectious pathogens like *P. multocida*, *G. parasuis*, swIAV, *S. suis* (Chae et al., 2021; Eddicks et al., 2021; Pieters and Maes, 2019). In addition, broncho-interstitial pneumonia lesions may also be scored based on the severity of the lesion (Calsamiglia et al., 2000; Sibila et al., 2012).

In addition to that, in early infections, one of the first physiological changes is the increase in body temperature (Arulmozhi et al., 2021; Burdick Sanchez et al., 2023). Assessment of the rectal temperature in *M. hyopneumoniae*-infected pigs is often used in experimental trials, but not much in the field. However, infrared cameras are being tested to monitor the body temperature of pigs in farm conditions (Jorquera-Chavez et al., 2021), with promising results, especially for more acute diseases. Some studies under experimental conditions reported the use of microchips to assess the body temperature (Lohse et al., 2010; Suli et al., 2017). However, when microchips are placed in different locations of the body (subcutaneous, intramuscular, or intraperitoneal), different results are obtained (Allerson and Morrison, 2011; Lohse et al., 2010).

1.3.6.2. Monitoring systems

New monitoring devices such as image sensors might be used to monitor clinical signs, and for early disease detection (Ahmed et al., 2015; Wei et al., 2023; Zhou et al., 2023). Besides being able to assess changes in behavior, image-based technology is evolving to evaluate the movement of grouped and individual animals.

Regarding movement, it is known that animals tend to decrease their activity when they are sick (Rostagno et al., 2011), making this information interesting when thinking about investigating the level of activity and movement in *M. hyopneumoniae*-infected and non-infected animals. A study using video-recording data (Escobar et al., 2007) showed

that PRRSV-positive pigs reduced overall activity. In the previous study, two groups were mixed to form the PRRSV-positive group, comprising pigs inoculated with PRRSV and pigs inoculated with both *M. hyopneumoniae* and PRRSV (Escobar et al., 2007). Although movement might be an indicator of disease, such information alone is not sufficient for diagnosis and should be combined with other parameters like molecular or serological tests.

1.3.6.3. Isolation of *M. hyopneumoniae* strains

Although isolation is described as the gold standard technique for diagnosing bacterial infections in general, *M. hyopneumoniae* (Chae et al., 2021), it is not widely used for *M. hyopneumoniae* because of its fastidious growth and frequent overgrowth of other bacteria (Chae et al., 2021; Pieters and Maes, 2019). Most medium preparations are still based on the formula and components described in 1975 by Friis (1975), which include, among other components, fresh yeast extract, porcine and horse serum, and phenol red, that serves as a pH indicator. Additionally, as its growth is very slow and other pathogens may grow earlier, specific antibiotics (i.e. kanamycin) can be added to the medium, especially to suppress *M. hyorhinis* growth (Cook et al., 2016; Kobisch and Friis, 1996). Ideally, the pathogen is isolated from lung tissue from the transition area of healthy-diseased tissue, but its isolation can also be attempted from BALF and swabs from the respiratory tract (Chae et al., 2021). A period of 3-4 weeks may be necessary to obtain an isolate, considering the time from lung sampling to identification of the isolate. Thus, unless isolates are needed, *M. hyopneumoniae* isolation is not routinely performed as a diagnostic tool.

1.3.6.4. Antigen and DNA detection

Immunohistochemistry, immunofluorescence assay, *in situ* hybridization, and PCR are examples of techniques applied to detect antigen or genetic material (DNA) of *M. hyopneumoniae* in tissue and other samples (Pieters and Maes, 2019). Immunohistochemistry and *in situ* hybridization allow identification and visualization of antigen and DNA, respectively. Antigen detection in fixed sections of tissue via

immunohistochemistry and immunofluorescence is based on the use of high-quality and specific labeled antibodies (mostly monoclonal) (Redondo et al., 2009), while *in situ* hybridization uses specific probes to detect specific nucleic acids (Garcia-Morante et al., 2022; Pieters and Maes, 2019). Even though *in situ* hybridization is more specific than immunofluorescence and immunohistochemistry, its use in routine diagnostic labs is not very common as it is more expensive (than immunohistochemistry) and the protocol itself is more complex and time-consuming than other available techniques like PCR, that also targets DNA (Chae et al., 2021; Pieters and Maes, 2019). Lastly, as the abovementioned techniques require tissue, their use to detect *M. hyopneumoniae* in live animals is limited.

1.3.6.5. PCR

Polymerase chain reaction tests can be performed on samples obtained from live animals (trachea-bronchial, laryngeal, and nasal swabs, bronchial mucus, oral fluids, BALF) and until now is the most commonly and sensitive technique used for *M. hyopneumoniae* detection worldwide. However, the selection of the sample should be made according to the purpose of the diagnosis, as false-negative results can occur more or less frequently, depending on the sample used (Marois et al., 2007; Pieters et al., 2017). When using oral fluids to detect *M. hyopneumoniae*, negative results should be interpreted critically, (Hernandez-Garcia et al., 2017; Pieters et al., 2017), as positive results are only seen in cases of clinical disease and high infection levels. Thus, the sensitivity and accuracy of the technique should be considered.

Several protocols have been described in the literature, some with single detection of the pathogen's DNA (Sibila et al., 2012; Stark et al., 1998; Strait et al., 2008), others that are able to detect different genes of *M. hyopneumoniae* (Marois et al., 2010), and others as multiplex (Fourour et al., 2018b; Stakenborg et al., 2006), which can detect different pathogens at once. Importantly, PCR was first used as a qualitative method (Stark et al., 1998), but has evolved to a quantitative technique (Fourour et al., 2018b; Marois et al., 2010; Sibila et al., 2012), allowing the quantification of bacterial DNA. Additionally, in the last few years, digital PCR has also been used to detect *M. hyopneumoniae* (Beuckelaere et al., 2022; Biebaut et al., 2023; Zhao et al., 2023), but

may also be used to easily detect different strains and genes in the near future. Digital PCR is an absolute quantification method that does not involve the generation of a standard curve, shows robust detection efficiency (Zhao et al., 2023), and greater sensitivity than real-time PCR and is more suitable for samples with a low bacterial load (Taylor et al., 2017).

Lastly, sequencing techniques may also be used for *M. hyopneumoniae* detection, although mainly used for whole-genome sequencing and metagenomic analysis. Next-generation (shotgun) (Wensel et al., 2022) and third generation (nanopore) (Wang et al., 2021) sequencing methods are widely used for microbiome analysis, being able to identify several microorganisms in a sample, measuring the abundance of each one of them. These analyses contribute to a better understanding of the associations between the respiratory microbiome and diseases (Blanco-Fuertes et al., 2021; Correa-Fiz et al., 2019; Espinosa-Gongora et al., 2016; Li et al., 2021; Niederwerder, 2017; Pirolo et al., 2021; Siqueira et al., 2017).

1.3.6.6. Antibody detection

Thus far, antibody detection is one of the most commonly used techniques (along with PCR) to investigate *M. hyopneumoniae* infection in pigs and is mostly performed using Enzyme-Linked Immunosorbent Assays (ELISA). Several assays are commercially available, mainly intended to detect specific immunoglobulin (Ig) G in serum samples (Pieters et al., 2017; Pieters and Maes, 2019), but detection of IgM and IgA is also possible (Gomes Neto et al., 2014; Matthijs et al., 2019b). The commercial assays are based on indirect and competitive ELISAs (Gomes Neto et al., 2014). In a study comparing different ELISA kits, Gomes-Neto et al. (2014) suggested that IgA responses should be further explored, as it can provide a sensitive, cost-effective surveillance tool, and may be able to distinguish vaccinated from infected animals (Bai et al., 2018). Meens et al. (2010) identified an infection-specific serological marker (Mhp366) that facilitates the detection of flourishing *M. hyopneumoniae* infections, and Bai et al. (2018) further worked on this discovery by developing an ELISA that seems capable to differentiate between infected and vaccinated animals. In addition to that, the authors have demonstrated that sera

cross-reactivity among all major swine mycoplasmas can occur. However, it should be further investigated, especially because of the small sample size in the study (Gomes Neto et al., 2014).

Seroconversion of infected animals has been reported to last for over 200 days (Pieters et al., 2009), with initial detection from 14 days pi onwards (Almeida et al., 2020c). In field conditions, seroconversion is very variable, mainly because animals can get infected at different moments, with different infection loads, but also because of the possible antigenic and virulence diversity observed in different isolates (Garza-Moreno et al., 2019; Vicca et al., 2003a). Thus, investigation of *M. hyopneumoniae*-specific antibodies should also consider that not all infected animals will seroconvert and that antibodies may not be detected for a certain period of time (Pieters et al., 2009; Vicca et al., 2003a).

1.3.7. Treatment and control

Antimicrobial treatment of *M. hyopneumoniae*-infected pigs is especially needed in outbreaks and to improve the health status of the herd, mostly preceding eradication programs, which are not yet common in countries like Belgium and Brazil. Because *M. hyopneumoniae* does not have a cell wall, commonly used antibiotics such as beta-lactams and polymyxins cannot be used (Maes et al., 2018c). As a consequence, a limited number of antimicrobials can be used, including tetracyclines, 15- and 16-membered ring macrolides, lincosamides, pleuromutilins, fluoroquinolones, florfenicol, aminoglycosides and aminocyclitols (Gautier-Bouchardon, 2021; Maes et al., 2018c; Stingelin et al., 2022). Tetracyclines (chlortetracycline, doxycycline, oxytetracycline, tetracycline) and macrolides (gamithromycin, tildipirosin, tilmicosin, tulathromycin, tylosin, tylvalosin) have been the most commonly used antimicrobials. Overall, experimental and field studies evaluated the impact of treatment on clinical signs, lung lesions, and in some cases, performance parameters, showing better results in the treated groups (Ciprian et al., 2012; Hannan et al., 1982; Mateusen et al., 2001; McKelvie et al., 2005; Stipkovits, 2002; Vicca et al., 2005). Administration of antimicrobial is done via the intramuscular (IM) or oral (water or feed) routes. When individual animals are acutely affected, the IM route is

preferred, but due to the chronic condition and high morbidity, antimicrobials are often administered via feed or water, intending metaphylaxis of a group (del Pozo Sacristán, 2014). Overall, the duration of treatment tends to be very long, varying from five to 21 days (del Pozo Sacristán, 2014). Lastly, according to the guidelines suggested by the knowledge center on AntiMiCrobial use and Resistance in Animals in Belgium, individual treatment should always be preferred instead of group treatment (AMCRA, 2022). If group treatment is needed, the veterinarian must conduct a clinical examination before resorting to such an approach.

Due to the rising concerns over antimicrobial resistance, alternatives such as vaccination and eradication are being considered (Maes et al., 2018c; Pieters and Maes, 2019). In this sense, it is also important to understand the factors affecting *M. hyopneumoniae* disease, which are mainly related to the production system (i.e. herd size), replacement animals (i.e. gilts mainly), management (i.e. stocking rate and density), and housing conditions (i.e. thermal conditions, air contaminants) (Marco et al., 2021). Nathues et al. (2013) observed that the risk for infection in weaning piglets was significantly increased when the number of purchased gilts per year and the number of farrowing pens per compartment increased. The group also showed a reduced chance of finding *M. hyopneumoniae* in suckling pigs in all in-all out farrowing systems when employing batch-wise farrowing every week or every three weeks (Nathues et al., 2013). Furthermore, controlling *M. hyopneumoniae* infection requires effective gilt acclimation before placing these naïve gilts into breeding herds, especially because vaccination cannot stop infection or shedding (Pieters and Maes, 2019). A study by Garza-Moreno et al. (2017) reported that vaccination against *M. hyopneumoniae* was the most used acclimation procedure (58.2%), followed by vaccination and animal exposure (21.3%) to other (likely positive) animals. Exposing naïve animals to the pathogen using lung homogenates is also effective and possible, but mainly used in the US (Garcia-Morante et al., 2016; Robbins et al., 2019). Temperature, air inlets/outlets, and location of fans's location, as well as air contaminants (i.e., ammonia and dust) also play a role in the development of respiratory disease in pigs. Michiels et al. (2015) reported a positive and significant association between particulate matter 10 (PM_{10}) and the extent of pneumonia

lesions, while ammonia was not associated with pneumonia lesions. Therefore, by improving/changing management conditions, such as the ones mentioned above, the risk of respiratory diseases will likely decrease.

As indicated above, vaccination plays a major role in the control of *M. hyopneumoniae*. Almost 70% of pig herds worldwide use commercial vaccines, which are composed of inactivated, adjuvanted whole-cell preparations (Maes et al., 2008). Improvement of daily weight gain, feed conversion ratio, shorter time to reach slaughter weight, reduced clinical signs and lung lesions, and lower treatment costs are among the benefits of vaccinating the animal (Maes et al., 1998; Pieters and Maes, 2019). Even though vaccination does not prevent infection, it reduces the number of organisms in the respiratory tract (Meyns et al., 2006) and decreases the infection level in the herd (Sibila et al., 2007). The specific protective mechanisms are still a matter of some debate. It has been demonstrated that injecting a commercial bacterin IM may lead to the production of IFN-γ secreting cells in blood as well as the secretion of certain antibodies in the respiratory tract and serum (Thacker et al., 2000), thus suggesting that cell-mediated immunity responses and local mucosal antibodies are critical for protection.

Besides the commercial vaccines, experimental formulations are constantly being tested (Marchioro et al., 2014; Matthijs et al., 2019a; Matthijs et al., 2019b; Mechler-Dreibi et al., 2021b), as summarized by Maes et al. (2021a; Maes et al., 2021b). Various vaccination strategies are employed based on factors like herd type, production system, and infection patterns (Maes et al., 2018c). Traditionally, double vaccination was more common, but nowadays, one-shot vaccines have gained popularity for providing similar benefits with less labor, making them more convenient for routine farm management (Maes et al., 2021a; Maes et al., 2008; Pieters and Maes, 2019). Additionally, the success of vaccination relies on several factors, like choosing suitable antigens, administration routes, and types of adjuvants and carrier molecules (Maes et al., 2018c). Thus, some practical considerations related to vaccine cost and administration, as well as potential combination with vaccines for other porcine pathogens, are crucial and should always be considered.

1.3.8. Eradication

Over the years, the successful elimination of *M. hyopneumoniae* from swine herds has been reported using various protocols (Gulliksen et al., 2021; Heinonen et al., 2011; Holst et al., 2015; Maes et al., 2008; Meszaros et al., 1986; Rautiainen et al., 2001; Yeske et al., 2021), including the Swiss method that involves partial depopulation and whole-herd medication (Zimmermann et al., 1989). While effective, this method is challenging for large herds. Alternative strategies like herd closure and medication, along with whole herd medication, have been developed (Holst et al., 2015), with the former allowing the introduction of replacement gilts early on (Pieters et al., 2009). A recent study by Yeske et al. (2020) reported that herd closure and medication resulted in longer periods without detection of *M. hyopneumoniae*, which is related to the lower hazard of bacterial detection. Whole herd medication was also associated with a higher hazard of detection of *M. hyopneumoniae* in the first months (Yeske et al., 2021). Nevertheless, ethical and economic justifications seem to support the elimination of *M. hyopneumoniae* from commercial herds, either alone or in combination with other pathogen elimination efforts (Yeske et al., 2021).

Chapter 2 | Knowledge gaps and scientific goals

Mycoplasma hyopneumoniae, the primary cause of EP, also contributes to the PRDC, which involves various infectious agents like PRRSV, PCV-2, *P. multocida*, among others. This emphasizes the extensive impact of *M. hyopneumoniae* on swine respiratory health and its potential role in disease interactions. Considering that the lung microbiome most likely plays a pivotal role in lung health, and that the knowledge on the pig lung microbiome is still very limited (Pirolo et al., 2023; Pirolo et al., 2021; Siqueira et al., 2017), more research is needed to understand the microbiome and the interactions between commensal microorganisms and respiratory pathogens.

Diagnosing *M. hyopneumoniae* infection involves assessing parameters like clinical signs (cough, fever), lung lesions, ADWG, air percentage in the lung, PCR, and ELISAs. Although commonly used, evaluating these parameters can be labor-intensive, time-consuming, and expensive, prompting questions about their utility or cost-effectiveness. This suggests that optimal diagnostic accuracy requires selecting the most pertinent parameters for assessment of the disease in *M. hyopneumoniae*-related research. Additionally, with an increasing need for real-time monitoring in pig farming, innovative technologies like sensors and artificial intelligence are being introduced to pig farming. This may enable the continuous monitoring of animal behavior, including movement, a possible indicator of health or welfare. In addition, it is not yet known whether *M. hyopneumoniae* infections affect movement of pigs.

Therefore, to address the above knowledge gaps, this thesis investigated the respiratory microbiome of *M. hyopneumoniae*-infected and *M. hyopneumoniae*-free pigs, and novel tools for continuous real-time monitoring of movement and body temperature as potential supportive tools in the diagnosis of *M. hyopneumoniae* infections. The specific objectives were:

- to investigate possible differences in microbe composition of the upper and lower respiratory tract of *M. hyopneumoniae*-infected versus *M. hyopneumoniae*-free finishing pigs (Chapter 3).
- i) to assess the impact of *M. hyopneumoniae* infection on the movement and the body temperature of piglets using a camera monitor and thermal microchips, and ii) to investigate the associations between movement, temperature, and

established parameters to evaluate *M. hyopneumoniae* such as severity of coughing and lung lesions and bacterial load (Chapter 4).

Chapter 3 | Influence of *Mycoplasma hyopneumoniae* natural infection on the respiratory microbiome diversity of finishing pigs

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Abstract

Mycoplasma (M.) hyopneumoniae interacts with the respiratory microbiota and facilitates colonization of other pathogens. The present study investigated the pulmonary and nasal microbiota of *M. hyopneumoniae*-infected and *M. hyopneumoniae*-free pigs. Sixty-six pigs from three commercial herds were selected at the end of the finishing phase: 44 originated from two *M. hyopneumoniae*-positive herds and 22 from a *M. hyopneumoniae*-negative farm. At the slaughterhouse, samples of nasal turbinate (NT) and bronchus-alveolar lavage fluid (BALF) were collected. DNA was extracted with a commercial kit and the infection status was confirmed by qPCR. All samples from the same herd were pooled, and next-generation sequencing based on the hypervariable region V3–V4 of the 16 s bacterial rDNA was performed. Data analysis included the taxonomic analysis, Alpha diversity indexes, and Principal coordinates analysis (Pcoa) using Jaccard, Bray–Curtis, Weighted Unifrac, and Unweighted Unifrac distances. All pigs from the infected herds tested PCR positive for *M. hyopneumoniae*, whereas all pigs from the negative farm were negative. There was a greater diversity of microorganisms in BALF when compared to NT samples in all the farms. BALF samples from infected animals showed higher abundance of *M. hyopneumoniae* than NT samples and a predominance of *Pasteurella multocida* among the main species identified, which was also abundant in the *M. hyopneumoniae*-free herd. PCoa diagrams indicated that for most of the samples, dissimilarity on bacterial composition was observed, regardless of infection status and sample type. Therefore, the lung microbiota was modulated by *M. hyopneumoniae* infection, which could play a role in the pathogenesis of *M. hyopneumoniae*-disease.

Keywords: *M. hyopneumoniae*, microbiota diversity, respiratory tract, lung lesion, swine

Introduction

Mycoplasmas are small bacteria without a cell wall that affect several animals, including pigs. Different species are known to infect swine, among them *Mycoplasma (M.) hyopneumoniae*, *Mycoplasma (M.) flocculare*, and *Mycoplasma (M.) hyorhinis*. *M. hyopneumoniae* is considered the primary agent of Swine Enzootic Pneumonia (SEP), a disease that results in chronic pneumonia, non-productive dry cough, growth retardation, and a negative impact on productive indicators (Pieters and Maes, 2019). Besides, *M. hyopneumoniae* can also play an important role in the Porcine Respiratory Disease Complex (PRDC), a multifactorial disorder where several infectious agents can be involved, such as porcine reproductive and respiratory syndrome virus (PRRSv), Porcine circovirus 2 (PCV2), and *Pasteurella* (*P.*) *multocida* (Pieters and Maes, 2019; Yaeger and Alstine, 2019).

Mycoplasma hyopneumoniae infections are commonly associated with a decrease in performance and an increase in costs related to control and treatment (Ferraz et al., 2020; Maes et al., 2008). The bacteria colonize the ciliated respiratory epithelium, promoting an exacerbated inflammatory response, which generates lesions that favor opportunistic pathogens, like *Actinobacillus (A.) pleuropneumoniae*, *Streptococcus (S.) suis*, *P. multocida*, and *Glaesserela (G.) parasuis* (Maes et al., 2008; Pieters and Maes, 2019).

Although considered commensal bacteria in the respiratory tract of swine, *M. hyorhinis* has been shown to be associated with *M. hyopneumoniae* infection and PRDC (Ferreira et al., 2021a; Pieters and Maes, 2019). In addition, it is a well-known cause of polyserositis and polyarthritis in piglets, and less often is associated with pneumonia, otitis, conjunctivitis, and abortion (Pieters and Maes, 2019). Recently, *M. flocculare*, another commensal bacterium in swine, has shown a negative correlation with the extension of MLCL when associated with *M. hyopneumoniae* infection (Ferreira et al., 2021a). Since the detection of one decreased the probability of detecting the other, it is possible that *M. hyopneumoniae* infection changes the environment in the respiratory tract, thereby creating unfavorable conditions for *M. flocculare* (Ferreira et al., 2021a; Fourour et al., 2018b).

A study by Huang et al. (2019) has shown higher abundance of *M. hyopneumoniae*, along with other bacterial genera (*Ureaplasma*, *Glaesserella*, and *Phyllobacterium*) in the microbiome of macroscopically affected lungs, when compared to healthy lungs, where the main genera reported were *Methylotenera*, *Prevotella*, *Sphingobium*, and *Lactobacillus* (Huang et al., 2019). Additionally, *M. hyopneumoniae* was positively associated with the extent of the lung lesions and reported as a taxonomic driver of functional shifts in the lungs with severe lung lesions (Huang et al., 2019). Other authors have also reported an apparent predominance of *M. hyopneumoniae*, *M. flocculare*, and *M. hyorhinis* in the lungs of pigs affected by enzootic pneumonia (Charlebois et al., 2014; Siqueira et al., 2017). Despite little information on the respiratory microbiota of pigs, and how the different species of microorganisms interact with each other, it is known that the diversity of bacterial species has been associated with greater stimulation of the immune system and strengthening of the non-specific immune response, both locally and systemically (Correa-Fiz et al., 2019). Thus, knowing how these interactions occur is essential to better address the control and prevention of coinfections, either bacteria-bacteria or bacteria-viruses (Abt et al., 2012).

As reported by Siqueira et al. (2017), *M. hyopneumoniae* was confirmed with a high prevalence when referring to community composition in lungs. Moreover, the authors suggest that the interactions among respiratory pathogens are numerous and complex, and therefore, can be additive or synergistic. In general, populations of microorganisms in organs of healthy individuals can help their functions and/or prevent colonization by pathogens. As an example, *Lactobacillus* spp. contribute to the formation of alveoli and mucus, and consequently they improve the functioning and protection of the respiratory tract (Yun et al., 2014).

Considering the abundance of *M. hyopneumoniae* and other bacterial species in the respiratory tract of pigs, the present study investigated possible differences between the pulmonary and nasal microbiota of finishing pigs naturally infected with *M. hyopneumoniae* and *M. hyopneumoniae*-free pigs in order to better understand the interactions and pathogenesis.

Materials and methods

Experimental design and sample collection

This study was submitted to the Ethics Committee in Animal Use of the Faculty of Agricultural and Veterinary Sciences of the São Paulo State University - Jaboticabal, and approved under protocol # 2032/21.

Three commercial swine herds (H), located in the state of São Paulo - Brazil, were selected for the study: one herd was free of *M. hyopneumoniae* (H1) and the other two were infected with *M. hyopneumoniae* (H2 and H3), as reported by the herd veterinarian and the presence of MLCL, suggestive of enzootic pneumonia. Moreover, the *M. hyopneumoniae*-status of the three farms was confirmed by testing bronchus-alveolar lavage fluid (BALF) and nasal turbinate (NT) samples with qPCR (Ferreira et al., 2021a; Fourour et al., 2018b). The free farm is a nucleus breeding herd of 650 sows, farrow-to-finish system, free of *A. pleuropneumoniae* and with vaccination against Influenza virus, Porcine Circovirus 2, Parvovirus, Leptospirosis, Erysipelas, and *G. parasuis*. The infected animals originated from two different commercial farms of 550 and 600 sows, farms H2 and H3, respectively. These two farms also used farrow-to-finish systems, with vaccination against *M. hyopneumoniae*, *P. multocida*, Influenza virus, Porcine Circovirus, Parvovirus, Leptospirosis, Erysipelas, and *G. parasuis.* The vaccination protocol is described in Additional file 1. Regarding antimicrobial use, although the animals from all farms were sporadically treated with antimicrobials in the feed until 75 days of age (amoxicillin, tiamulin, doxycycline and/or florfenicol), they did not receive any antimicrobial treatment for at least 2 months before slaughter. Natural ventilation and concrete floor with water blades were used for the finishing pigs in all farms. Moreover, all animals were from the same breed.

From each farm, 22 pigs (140-day-old average) were randomly selected and identified by an ear tag in the morning. The pigs were sent to the slaughterhouse later on the same day. Animals from the same farm were kept in the same rest pen for at least 3 hours before slaughter, and animals from H2 and H3 were not mixed with the *M. hyopneumoniae*-free pigs (H1). However, the rest pens were located in the same space, side by side, and separated by 1.5 m height concrete walls. Animals with *M.*

hyopneumoniae-free status were slaughtered first (H1), followed by the pigs from the *M. hyopneumoniae*-infected farms (H2 and H3).

At the slaughterhouse, after the scalding tank and during evisceration, the lungs and the corresponding head from the selected carcasses were separated for evaluation and sampling. The carcass and the nose of the pigs were sawn off in the slaughter-line using a saw (disinfected by water at 90 °C) to access the NT. Fragments of the NT were collected directly from the carcass using sterile forceps and scalp. Then, the fragments were inserted into cryotubes free of DNAse and RNAse (Corning, USA) and stored in a freezer at - 80 °C.

BALF was collected using a plastic pipette and automatic pipettor. For insertion of the pipette, an incision was made in the trachea, about 5 cm before the bifurcation of the bronchi. Directly into the bronchi, 20 mL of PBS (1X, pH 7.4; Sigma-Aldrich, Germany) was dispensed, followed by a gentle massage through the lung parenchyma and aspiration with a pipette, recovering between 10 and 15 mL of lavage. The recovered content was transferred to 50 mL falcon tubes (Corning, USA) and later aliquoted into 2 mL cryotubes free of DNAse and RNAse (Corning, USA), which were then stored in a freezer at -80 °C until further processing.

Macroscopic lung lesion scoring

At the time of slaughter, the lungs of the study pigs were removed from the carcass and the macroscopic lung lesions compatible with SEP were scored according to the methodology described by Straw et al. (1986). The apical, cardiac, accessory, and diaphragmatic lobes were evaluated separately. The scores could range from 0% (no lesions) to 100% (entire lung affected).

DNA extraction

BALF samples were thawed and centrifuged at 4 °C (Centrifuge 5804 R, Eppendorf, Germany) at 12 000 *g* for 20 min. After centrifugation, the supernatant was discarded and the pellet was used for DNA extraction. A total of 0.05 g of NT and the pellet from BALF samples were individually submitted to commercial DNA extraction using

the DNeasy blood and Tissue kit (Qiagen, USA), following the manufacturer's instructions. The extraction product was evaluated by spectrophotometry in Nanodrop® 2000c (Thermo Fisher, USA) to measure concentration and purity.

qPCR targeting the M. hyopneumoniae p102, M. hyorhinis p37 and M. flocculare fruA gene fragments

Gene fragments of *M. hyopneumoniae (p102), M. hyorhinis (p37),* and *M. flocculare (fruA)* from DNA samples were quantified by qPCR as described elsewhere (Fourour et al., 2018b), with modifications (Ferreira et al., 2021a). The standard curve with serial dilutions of constant ratio equal to 10 (10^7 to 10^1) was based on a synthetic DNA (GBlock®, IDT, USA) containing fragments of 150 base pairs (bp) (*p102*), 101 bp (*p37*) and 119 bp (*fruA*), used as positive control (Ferreira et al., 2021a). All samples were tested in duplicate, and the results were only accepted for those with a standard deviation lower than or equal to 0.5 cycle (Bustin et al., 2009). Samples with a deviation greater than 0.5 were retested in triplicates.

Pooling and amplification of bacterial 16s rDNA

The extracted DNA samples from animals of the same herd (H1, H2, or H3) were grouped in equimolar amounts according to the concentration (ng/µL) obtained by spectrophotometry. The pools from the control group were named H1NT and H1BALF, while the pools of samples from infected farms were named H2NT, H2BALF, and H3NT, H3BALF. After pooling and before submission to Illumina sequencing, the integrity and quality of the samples were evaluated using Bioanalizer® (Agilent -CA, USA).

Pooled DNA samples were submitted to a PCR targeting the hypervariable region V3-V4 of the 16s bacterial rDNA, using a previously described protocol (Klindworth et al., 2013), which flanks an amplicon of 465 bp. Then, Illumina sequences were added to the original primer sequence following the manufacturer's instructions. After amplification, integrity and production were evaluated by electrophoresis on a 2% agarose gel (Sigma-Aldrich, Germany) stained with SYBR gold (0.5 µg/mL) (Invitrogen®, USA). The primer sequences are described in the table below.

Table 2. Primer sequences used for amplifying the V3-V4 region of the 16S rDNA.

ID	Sequence (5'-3')
Forward	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGC WGCAG
Reverse	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGC WGCAG

Library preparation and sequencing

Library validation was performed in a Bioanalyzer 2100 (Agilent Technologies, USA) and quantified using the Kappa Library Quant Kit for Illumina (Illumina, USA), following the manufacturer's instructions. The library was then adjusted for equimolar concentration according to the manufacturer's instructions (Illumina., 2013). Then, 5 µL of each diluted library were mixed in a pool and submitted to Next-Generation Sequencing (NGS).

Sequencing was performed using the MiSeq Reagent Kit v3 (600 cycles) (Illumina, USA) on an Illumina MiSeq platform (Illumina, USA), according to Caporaso et al. (2012). The generated sequencing data were demultiplexed using Illumina bcl2fastq software (v2.19.1.403), and sequencing quality assessment was performed using DADA2 software (Callahan et al., 2016). Briefly, adapter sequences and end low quality reads were trimmed, removing the first 15 nucleotides of reads (forward and reverse) and cutting read ends at position 270. Trimmed reads were then filtered out with more than two expected errors. Both pairs of reads (forward and reverse) were removed when one was discarded. Then, DADA2 ran denoising by collapsing together all reads that encoded the same sequence and merged forward and reverse reads using alignment. Pairs of reads that did not match were discarded. Lastly, DADA2 removed chimera sequences. The parameters used for DADA2 analysis were: --p-trim-left-f 15 --p-trim-left-r 15 --p-trunc-len-f 270 --p-trunc-len-r270.

Data analysis

Correlations between MLCL and quantification of each *Mycoplasma* spp. were evaluated according to the normality of the data, where Spearman's Rank correlation test was used for non-parametric data ($p < 0.05$). To measure the strength of dependence between two variables, the Kendall' rank correlation test was used ($p < 0.05$). The R software version 3.5.1 (R Core Team, 2018) was used for data analysis.

The contigs were aligned and assembled in silico using the software Quantitative Insights Into Microbial Ecology - QIMEE version 2019.10 (Caporaso et al., 2011) and the SILVA database (release 132), reducing to the level of phyllo, family, and genera. For the analysis of bacterial diversity, a phylogenetic tree was constructed using the Mafft software for multi-sequence alignment and the FastTree software, both following the standard parameters. For alpha diversity's analyses, the following metrics were evaluated: OTUs quantity, Faith, Shannon, Pielou, and Simpson indexes. Main coordinates analysis was performed to detect similarities between uninfected (H1) and infected herds (H2 and H3), based on the following metrics: Jaccard, Bray-Curtis, Weighted Unifrac, and Unweighted Unifrac. For each metric, differences between infection status and/or tissue were calculated using multivariate permutational analysis of variance (PERMANOVA) at the significance level of $p < 0.05$.

Results

Mycoplasma detection and quantification in NT and BALF samples

The efficiency of qPCR reactions varied from 92.3% to 101.4%, R2 ranged from 0.992 to 0.999; the slope varied from −3.387 to −3.522. Detailed parameters are shown in Additional file 2. All BALF samples from H1 were negative for *M. hyopneumoniae* on the qPCR while 21 samples (95.4%) from H2 and all samples (100%) from H3 were positive. For NT, all samples from H1 were *M. hyopneumoniae*-negative while 8 samples (36.4%) from H2 and 4 samples (18.2%) from H3 were *M. hyopneumoniae*-positive. One animal in H2 tested negative for *M. hyopneumoniae* in the BALF sample and tested positive in the NT sample, and therefore, is considered *M. hyopneumoniae*-positive. Among all NT samples, 81.8% (54/66) tested positive for *M. flocculare* and 59.1% (39/66)

were positive for *M. hyorhinis*. Similarly, 80.3% (53/66) and 53% (35/66) of the BALF samples tested positive for *M. flocculare* and *M. hyorhinis*, respectively.

Median quantification values for *M. hyopneumoniae* in BALF and NT were 8.73 × 10^5 copies/µL (2.98 × 10^2 to 4.51 × 10^7) and 4.20 × 10^2 copies/µL (9.51 × 10^0 to 4.20 × 10^3), respectively. Detailed information regarding quantification data is summarized in Additional file 3. In total, 17 samples could not be quantified due to inconsistent quantification results (Cq values with a difference >0.5), even when tested in triplicate. This fact is likely due to the Markov Chain Monte Carlo effect (Bustin et al., 2009), which represents an inherent limitation of the qPCR technique, mainly in samples with a low number of DNA copies. Even though these samples could not be quantified, they were considered positive.

Correlations with Mycoplasmas quantification and macroscopic lung lesion score

The mean macroscopic lung lesion scores obtained from the different herds were: H1 – 0.5225 (SD = 2.58); H2 – 6.6025 (SD =14.34); and H3 – 2.7075 (SD = 6.25). Significant differences in the mean macroscopic lesion score were detected between *M. hyopneumoniae*-positive (H2 and H3) and *M. hyopneumoniae*-negative (H1) farms ($p < 0.05$). MLCL occurrence for H1, H2, and H3 was 68.18%, 90.9%, and 90.9%, respectively.

Spearman's correlation showed a significant positive correlation ($r = 0.62$; $p < 0.05$) between the quantification of *M. hyopneumoniae* in the BALF by qPCR and the macroscopic lung lesions. No significant correlations were observed between macroscopic lung lesions and *M. hyorhinis* or *M. flocculare* quantification results in BALF samples ($p > 0.05$). There were no significant correlations between macroscopic lung lesions and *M. hyopneumoniae*, *M. hyorhinis*, or *M. flocculare* quantification results in NT samples ($p > 0.05$).

Kendall's rank correlation test showed a negative correlation between the quantification of *M. hyopneumoniae* and *M. flocculare* in BALF ($r = -0.43$; $p < 0.05$), and no significant correlation between the quantification of *M. hyopneumoniae* and *M. hyorhinis* ($p = 0.89$). There was a significant positive correlation between the quantification of *M. hyopneumoniae* in BALF and NT samples ($p < 0.05$; rho = 0.4).

NGS results and quality assessment

The NGS identified a total of 4364 features or operational taxonomic unit (OTUs) with sequence sizes ranging from 255 bp to 498 bp, and an average sequence size of 377.7 bp (± 68.8). The OTUs and readings obtained for each pool before rarefaction are shown in Table 3.

Diversity and species richness in NT and BALF samples

To estimate the species diversity in the samples, the sequences were rarefied to a depth of 69 100 reads. The samples showed varying numbers of reads, with H2NT and H2BALF showing the highest values, followed by H3 samples, both from infected herds (Table 3). The higher sequencing throughput in both H2 samples was reflected in higher OTU values. A high OTU value was also observed for the H3NT sample, however, the H3BALF sample presented a low OTU value, resulting in lower diversity values. The H1BALF sample had a lower number of OTUs, possibly due to the low sequencing throughput (181.176 reads). Detailed information is presented in Table 3.

Table 3. NGS data and quality control obtained from pooled NT and BALF samples of the three farms.

Group	Sample type	Sample ID	OTUs	Number of reads
***M. hyopneumoniae*-free (H1)**	NT	H1NT	790	590 252
	BALF	H1BALF	182	181 176
***M. hyopneumoniae*-positive (H2 and H3)**	NT	H2NT	1384	1 242 750
		H3NT	1218	705 578
	BALF	H2BALF	1585	1 914 416
		H3BALF	481	1 229 248

Regarding the alpha diversity metrics calculated for each pool, the OTU values for NT samples were numerically higher (average: 987), indicating, in general, a greater

diversity of genders when compared to BALF samples (average: 572.6). In addition, Shannon values for BALF samples from infected animals were numerically lower (H2: 2.77 and H3: 1.68), indicating low diversity. Considering the uniformity aspect by the Pielou and Simpson metrics, the samples H2BALF and H3BALF showed the lowest values, indicating less uniformity between genera in the sample (Table 4).

Table 4. Alpha diversity metrics obtained from pooled NT and BALF samples of the three farms.

Group	Sample ID	OTUs	Faith	Shannon	Pielou	Simpson
***M. hyopneumoniae*-free** (H1)	H1NT	710	356.93	3.37	0.35	0.64
	H1BALF	182	63.08	3.79	0.50	0.87
***M. hyopneumoniae*-positive** (H2 and H3)	H2NT	1,120	533.08	4.18	0.41	0.79
	H3NT	1,131	552.68	5.04	0.49	0.84
	H2BALF	1,200	134.61	2.77	0.27	0.58
	H3BALF	336	122.79	1.68	0.20	0.48

Principal coordinates analyses (PCoa) of NT and BALF

Bray-Curtis dissimilarity distance

The PCoa constructed based on the Bray-Curtis distance showed that there was no separation between samples from different herds in any of the axes. Considering the PCoa analysis for tissue, it was observed that samples from different tissues appear separated in axis 1, with no cluster formation, but certain proximity between H2BALF and H3BALF, and greater dissimilarity in relation to the other samples (Figure 1).

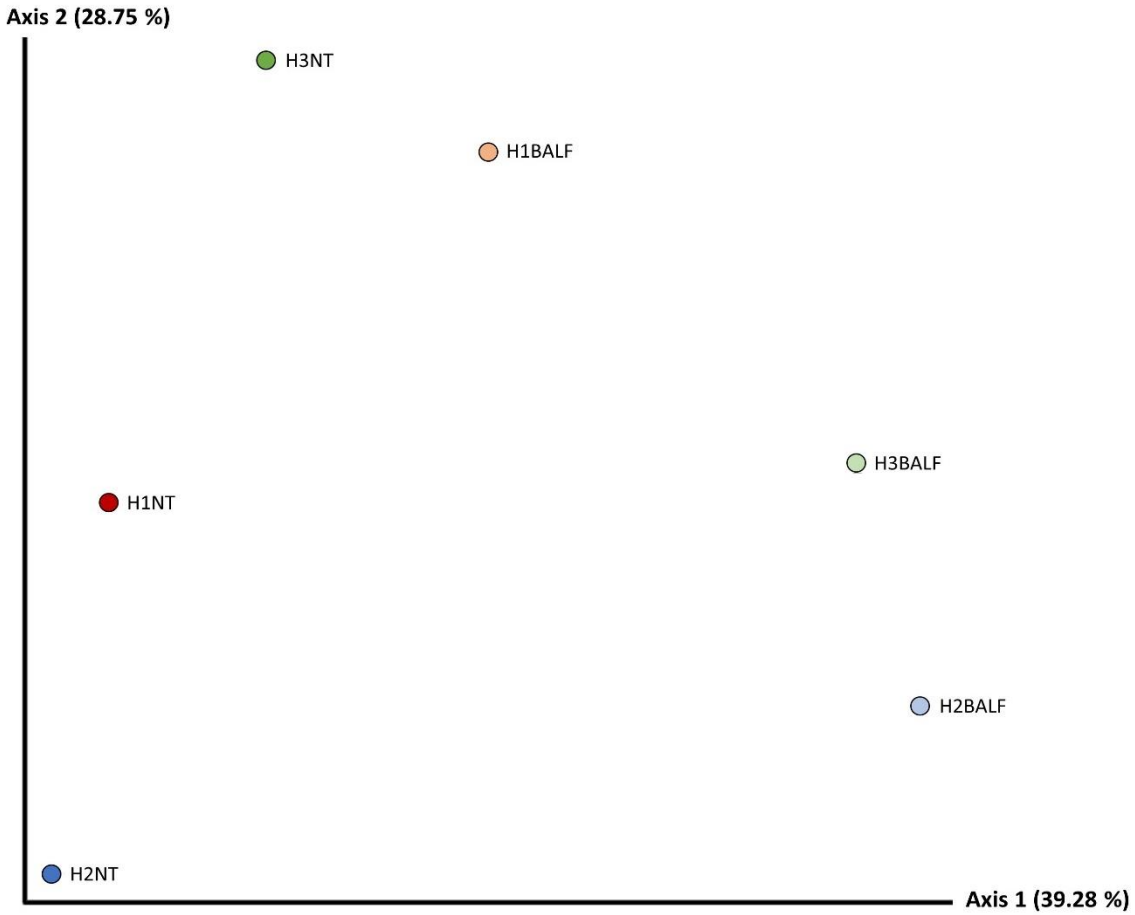

Figure 1. Bray-Curtis dissimilarity distance Pcoa diagram based on NT and BALF pooled samples of the *M. hyopneumoniae*-infected (H2 and H3) and *M. hyopneumoniae*-free (H1) herds.

Jaccard distance

The PCoa based on Jaccard's distance did not show proximity between any samples. Visually, a greater dissimilarity was observed for the H2BALF sample in relation to the others (data not shown).

Unweighted Unifrac distance

The PCoa based on the unweighted Unifrac distance showed separation between BALF and NT samples, with greater dissimilarity for NT samples on axis 1. The H2NT and H3NT samples were close to each other, indicating qualitative similarity between the

bacterial types present in both samples. Regarding BALF samples, no proximity was observed between any of the points regardless of the herd, with the greatest distance observed between H2BALF and H1BALF (Figure 2).

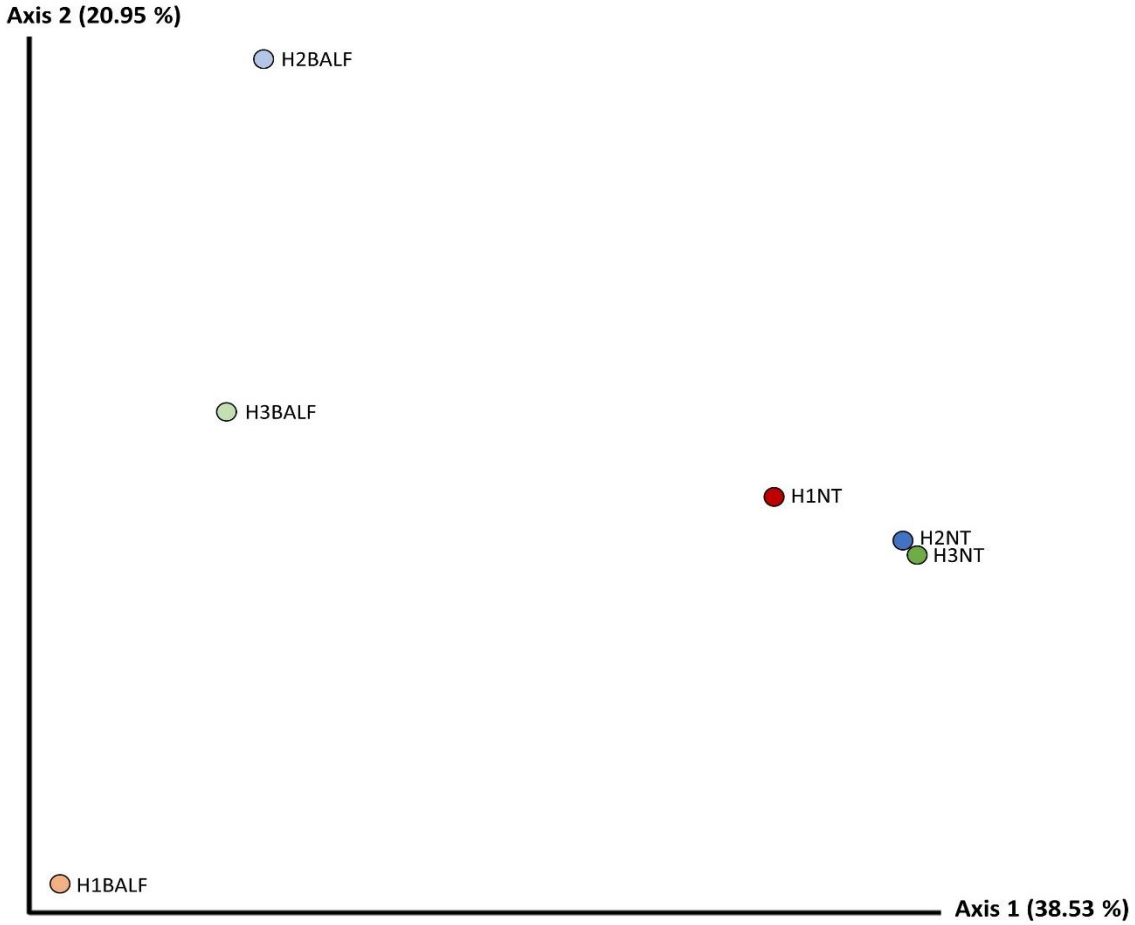

Figure 2. Unweighted Unifrac distance Pcoa diagram based on NT and BALF pooled samples of the *M. hyopneumoniae*-infected (H2 and H3) and *M. hyopneumoniae*-free (H1) herds.

Weighted Unifrac distance

In the weighted Unifrac distance PCoa, axis 1 explains a high percentage of variation in relation to the other axes (64.68%). The samples of H2BALF, H3BALF, and H1BALF were close to each other and relatively far from H1NT and H2NT samples, and even further away from H3NT, possibly due to the low abundance in the sample. NT and

BALF samples from uninfected animals were closer to each other when compared to corresponding samples in herds 2 and 3 (H2 and H3), as observed in Figure 3.

Figure 3. Weighted Unifrac distance Pcoa diagram based on NT and BALF pooled samples of the *M. hyopneumoniae*-infected (H2 and H3) and *M. hyopneumoniae*-free (H1) herds.

Taxonomic analysis

The taxonomic analysis showed a high relative frequency of *M. hyopneumoniae* in samples H2BALF and H3BALF (62.39% and 68.61%). *M. hyopneumoniae* was also detected in H1NT, H2NT, and H3NT, with the respective frequency of 0.15%, 0.09%, and 0.08%. Considering that all BALF and all NT samples were negative on the qPCR, it is possible that the occurrence of *M. hyopneumoniae* in H1NT is related to contamination during slaughter. In samples H2BALF, H3BALF, and H1BALF, relative frequencies of *P. multocida* were 18.21%, 4.83%, and 22.5%, respectively. *Anoxybacillus* was recorded in BALF and NT samples only in H3 and H1, ranging from 8.36% (H3BALF) to 36.36% (H3NT). Following the order of relative frequency, *Actinobacillus indolicus* appears in all

samples, but only in H2NT, a high relative frequency of 40.2% was recorded, and in the others, the values (H3: 0.58% and H1: 1.74%) were not higher than 2%. Cilia-associated respiratory bacterium 95-15405 was identified in samples from H1 and H2, with the highest relative frequencies observed in H2NT and H2BALF, 21.36% and 7.28%, while in samples from H1 the values were not higher than 4% (H1NT: 3.72% and H1BALF: 0.94%). Unexpectedly, a high relative frequency of 67.40% of *Escherichia/Shigella* was identified in the H1NT sample while samples H1BALF, H2BALF, and H3BALF showed relative frequencies close to 0.3%. *Mycoplasma flocculare* was identified in all analyzed samples, and among the BALF samples the highest value was for H1BALF (12.09%) and in lower prevalence in the samples H2BALF (0.5%) and H3BALF (0.18%). On the other hand, NT samples showed a higher prevalence of *M. flocculare* in H2NT (3.41%) and H3NT (12.68%), while in H1NT the percentage was the lowest, 1.48%. Main results are shown in Figure 4.

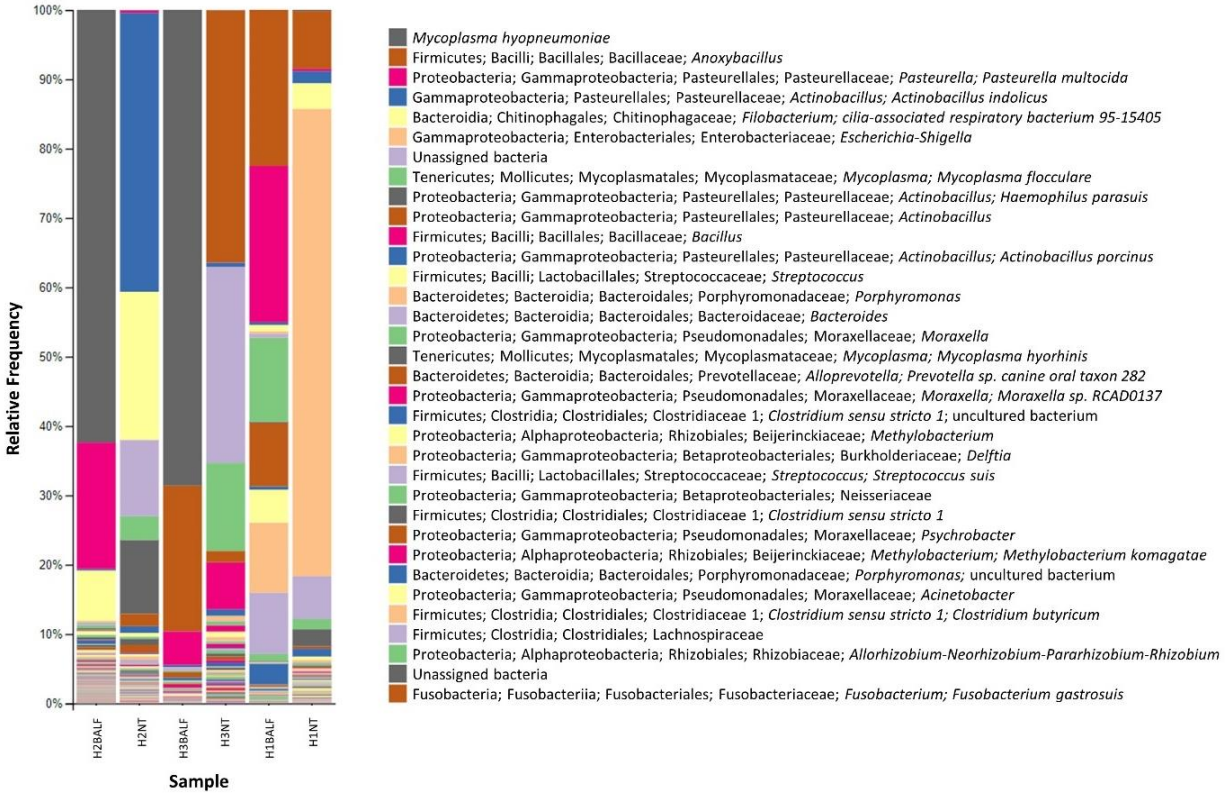

Figure 4. Taxonomic analysis based on NT and BALF pooled samples of the *M. hyopneumoniae*-infected (H2 and H3) and *M. hyopneumoniae*-free (H1) herds.

Discussion

Mycoplasma hyopneumoniae is described as the primary agent in respiratory diseases in pigs, present worldwide, and directly related to the occurrence of pulmonary consolidation lesions in pigs (Maes et al., 2018a). The present comparative study, carried out with nasal turbinate (NT) and bronchoalveolar lavage fluid (BALF) samples of animals infected and not infected by the pathogen, indicated a predominance of *M. hyopneumoniae* and low species diversity in alpha and beta diversity metric analyses, in addition to significant correlations between *M. hyopneumoniae* DNA quantification and MLCL.

The qPCR results showed a high occurrence of *M. hyopneumoniae* in H2 and H3 and high correlation with MLCL, which was also recently reported by Ferreira et al. (2021a). In addition, the authors indicated that the greater the extent of MLCL, the greater

the concentration of *M. hyopneumoniae* and, interestingly, the lower the concentration of *M. flocculare* in the lungs. Similarly, our results indicated that *M. hyopneumoniae* concentration in BALF is negatively correlated to *M. flocculare* concentration in BALF. Therefore, since these two *Mycoplasma* species share most of the surface proteins (Siqueira et al., 2014) and are closely related in phylogenetic analyses, it is possible that, during infection, antibodies against *M. hyopneumoniae* cross-react with *M. flocculare* antigens, which could reduce the number of microorganisms. Considering its lower capacity of evading the immune system, *M. flocculare* would be more susceptible to be eliminated in case of coinfections with *M. hyopneumoniae* (Ferreira et al., 2021a).

Not surprisingly, MLCL occurrence was higher in the infected herds than in the *M. hyopneumoniae*-free herd. The lesions were more severe in H2 than in H3, although *M. hyopneumoniae* relative frequency was higher in H3. However, *Pasteurella multocida* relative frequency was also higher in H2, which could have worsened the lung lesions in H2, as reported previously (Amass et al., 1994a; Ciprián et al., 1988; Smith et al., 1973). *P. multocida* is a secondary pathogen that in combined infections with *M. hyopneumoniae* results in more severe pneumonia and clinical disease (Fablet et al., 2012; Lopez Rodriguez et al., 2020; Maes et al., 2018c; Pieters and Maes, 2019; Smith et al., 1973). Besides, MLCL values in H1 were significantly lower than H2 and H3 even though the relative frequency of *P. multocida* was higher. Although our results suggest that *M. hyopneumoniae*-like lung lesions were worsened when *P. multocida* coinfection occurred, coinfections by other respiratory pathogens such as Influenza and PCV2 cannot be excluded as they were not assessed in this study.

Regarding the animals infected by *M. hyopneumoniae*, a predominance of the species in high relative frequency in BALF samples was shown, as well as lower numerical values in the diversity and uniformity metric, measured by the Pielou and Simpson analyses. Numerically, the H1BALF sample showed better results considering uniformity, according to Pielou and Simpson metrics, although Faith and OTUs values were low, possibly due to the low sequencing throughput (181.176 reads). Accordingly, previous studies have shown that animals infected with *M. hyopneumoniae* have a predominance of the species over other species, reducing bacterial diversity in the lung (Charlebois et

al., 2014; Palzer et al., 2008). It is known that *M. hyopneumoniae* stimulates the immune system, generating the release of various pro-inflammatory cytokines (Almeida et al., 2020a; Choi et al., 2006; Mechler-Dreibi et al., 2021a; Woolley et al., 2012), induces apoptosis (Bai et al., 2013), nitric oxide stimulation (Hwang et al., 2011), which promote lung damage in infected individuals. Likewise, lung damage was also observed in the present study, as animals positive for *M. hyopneumoniae* had a higher degree of pulmonary involvement. Therefore, it is possible that the indirect effects on lung injuries could be associated with the reduction in the diversity of local microorganisms, either pathogenic or commensal.

Microbiome studies have demonstrated the beneficial effect of the diversity and uniformity of bacterial species, as well as the protective benefits the presence of certain species may have for the host (Li et al., 2021). In our study, lower diversity values were observed in BALF samples of *M. hyopneumoniae*-infected animals, which may be an indicator of the lung's health since higher diversity was observed in the non-infected animals (H1BALF). Other studies have shown that bacterial diversity helps to maintain balance by preventing the spread of pathogens and susceptibility to chronic diseases (Buffie and Pamer, 2013; Karkman et al., 2017). In healthy and diverse microbiota, pathogenic bacteria are less likely to prevail due to competition for space, nutrients, and host receptors (Reid et al., 2001).

BALF samples from *M. hyopneumoniae*-infected pigs showed similarities in relation to the high abundance of *M. hyopneumoniae*, and the predominance of *P. multocida* among the main species of bacteria identified. Moreover, it seems that other bacterial genera are present in both infected and *M. hyopneumoniae*-free BALF samples due to the similarity observed in the PCoa metrics by Bray Curtis. *M. hyopneumoniae* evasion characteristics and its ability to induce local inflammation are strategies that allow the pathogen to be present at higher relative frequencies (Almeida et al., 2020a; Maes et al., 2018c; Pieters and Maes, 2019). Additionally, it seems to be the predominant species in the lungs of infected animals, which could indicate a way to perpetuate the infection and reduce competition for adhesion sites. However, additional studies are needed to better evaluate this hypothesis.

In contrast to the BALF results, low relative frequencies of *M. hyopneumoniae* were detected in NT samples, as well as better results from the point of view of alpha diversity. This corroborates literature data that state that *M. hyopneumoniae* presents tropism for the lower parts of the respiratory tract of pigs (Marois et al., 2007; Otagiri et al., 2005). Surprisingly, *M. hyopneumoniae* was identified, in a very low relative frequency, in the H1NT sample, but not in H1BALF or the individual qPCR results. Even though *M. hyopneumoniae* was identified in NT sample from a negative farm, it is possible that cross contamination occurred during the slaughter process, as reported by Marois et al. (2008), where *M. hyopneumoniae* DNA was found in nasal and tracheal swabs of SPF pigs that had no contact with other pigs at slaughter. In that case, the scalding water could have been a source of contamination since *M. hyopneumoniae* DNA was detected even before the onset of slaughter (Marois et al., 2008). The authors also reported a higher occurrence of *M. hyopneumoniae* DNA in SPF pigs that were in the rest area for 4 hours before slaughter. Therefore, considering the similar conditions in our study, it is possible that infection occurred just before slaughter in the rest pen, when *M. hyopneumoniae*-infected and non-infected pigs were allocated close together, or during the slaughter process.

Qualitative measures of dissimilarity, Jaccard, and Unweighted unifrac, observed in the PCoa diagrams indicated that for most of the samples, dissimilarity was observed, regardless of infection status by *M. hyopneumoniae* and sample type, with the exception of H2NT and H3NT samples that were relatively close to each other, indicating similarity between the compositions of microbiota in these samples. Although most of the points are equidistant, it was possible to notice in the PCoa diagram based on the Unweighted unifrac distance, that the distance was greater between BALF and NT samples. The greatest dissimilarity was registered between H1BALF and H2BALF, indicating qualitative differences between bacteria that make up both samples. In addition, the composition and diversity of the microbiota also seem to be closely related to environmental conditions. Differences in the swine rearing environment have been shown to have an influence on the animals' respiratory and intestinal microbiota (Megahed et al., 2019; Schmidt et al., 2011). Therefore, when establishing comparisons between animals from different

properties, it is necessary to take into account the fact that they may have been colonized by different bacteria, which could result in lack of beta similarity between samples.

Furthermore, the microbial composition of pig's microbiota is dynamic and is related to several factors, such as environment, production system, diet, antimicrobial use and others (Correa-Fiz et al., 2019). The impact of antimicrobial use on the microbiota of pigs depends on the chemical structure, route of administration, dose, and duration of treatment (Ferrer et al., 2017). Zeineldin et al. (2018) reported that each of the different antimicrobials tested in their study had a distinct impact on the nasal microbiota community structure, and that short-term antimicrobial administration could alter the nasal microbiota diversity and richness. Additionally, Correa-Fiz et al. (2019) noted a lower relative abundance of potentially pathogenic bacteria in the nasal microbiota, while beneficial bacteria were more abundant in the farms where no perinatal antimicrobial treatment was used. Nevertheless, considering that the respiratory microbiota normally reaches stability 2-3 weeks after weaning and that the effect of antimicrobial treatment may differ in adult animals with an established nasal microbiota, further studies aiming at exploring the effects of antimicrobial use on the respiratory microbiota of healthy and clinically affected pigs should be conducted (Zeineldin et al., 2018).

In general, the abundance of microorganisms in the lung microbiota was affected by *M. hyopneumoniae* infection in BALF pools, but not in NT samples. The beta diversity analyses indicated differences between the abundance of microorganisms between the BALF samples when comparing *M. hyopneumoniae*-infected and non-infected herds, which is confirmed by observing the taxonomic analysis. Therefore, it is possible to observe different bacteria with approximate relative frequencies, which makes it more difficult to determine a single predominant species. On the other hand, samples H2BALF and H3BALF demonstrated the predominance of *M. hyopneumoniae*. Nonetheless, further studies comparing the impact of different *M. hyopneumoniae* strains on the lung microbiota of infected animals are needed, as little is known about the virulence of the strains circulating in Brazil (Minion et al., 2004; Takeuti et al., 2017).

In our study, sequencing pooled samples was a determining factor in making it possible to have an overview of the respiratory microbiota from the three groups,

especially when considering the costs of the analyses. As shown by Ray et al. (2019), pooling samples can cut the costs 10-fold. In addition, pooling samples before DNA amplification to estimate community level diversity is a viable and valuable measure to consider in population-level studies (Ray et al., 2019). However, pooling the individual samples to one sample per farm has the disadvantage that no quantitative comparisons and statistical analyses can be made due to the small number of observations.

Although our results are important and can be useful to help understanding the respiratory microbiota modulation in *M. hyopneumoniae*-infected pigs, some experimental limitations should be addressed and acknowledged, and the results reported here should be interpreted with caution. For instance, although NT samples showed higher diversity, the detection of *M. hyopneumoniae* was lower for these samples. Therefore, considering our results, the use of BALF samples seems more valuable when the aim is to assess possible associations between *M. hyopneumoniae* infection and the respective influence on the microbiota. Additionally, since sampling collection took place in the slaughterhouse, some sources of contamination such as the scalding water and the sawing machine were controlled only by the federal inspection system, and not by the researchers. Thus, possible contamination in the upper respiratory tract samples needs to be taken into account when interpreting the respective results. Even though internal negative controls collected at sampling and at DNA extraction were included and tested negative by qPCR for *Mycoplasma* (Ferreira et al., 2021a), these samples were not sequenced and therefore, a possible background contamination in the microbiome analysis cannot be ruled out. However, it is important to mention that all samples were processed in the same way, using the same procedures and using reagents from the same batch.

Overall, it has been observed that the presence or absence of a particular pathogen can affect directly or indirectly, not only promoting the development of lesions but also modulating the diversity, uniformity, and abundance of other bacterial populations in the respiratory tract of pigs. However, since the animals were assessed at slaughter, the results are not necessarily applicable for the situation during fattening period and earlier stages. Therefore, our results partially demonstrate the impact of *M. hyopneumoniae* in the lungs of infected pigs at slaughter, and further studies are needed to investigate the

significance of *M. hyopneumoniae* infection in the microbiome of diseased pigs in comparison to healthy animals.

The presence of *M. hyopneumoniae* in BALF of slaughter pigs seems to modulate the microbiota, which could facilitate dysbiosis and proliferation of other pathogens. *M. hyopneumoniae* quantification was strongly correlated with macroscopic lung consolidated lesions, meaning that the severity of the lesions increases when there is a higher number of microorganisms.

Additional files

Additional file 1. Detailed vaccination protocol for farms H1, H2, and H3.

	Piglets in nursery		Sows
	1st dose (21 days)	**2nd dose (42 days)**	**5 to 10 days post-partum**
M. hyopneumoniae*	X	X	
P. multocida	X	X	
G. parasuis	X	X	
Influenza	X	X	
Porcine Circovirus 2	X	X	
Parvovirus			X
***Leptospira* sp.**			X
E. rhusiopathiae			X

*Vaccination only in farms H2 and H3.

Additional file 2. Parameters for qPCR assays based on the gene fragments of *M. hyopneumoniae (p102), M. hyorhinis (p37),* and *M. flocculare (fruA).*

	Fluorophore (gene)	Efficiency (E)	r^2	Slope	Y-intercept
Plate 1	FAM (*p102*)	98.5	0.998	-3.357	40
	Cy5 (fruA)	97.1	0.995	-3.393	39.7
	Texas Red (p37)	100.4	0.995	-3.313	37.7
Plate 2	FAM (*p102*)	101	0.998	-3.299	40.5
	Cy5 (fruA)	99.3	0.992	-3.339	38.8
	Texas Red (p37)	101.4	0.999	-3.29	38.9
Plate 3	FAM (*p102*)	97.8	0.998	-3.376	40.8
	Cy5 (fruA)	101.5	0.996	-3.287	38.7
	Texas Red (p37)	100.9	0.997	-3.3	38.6
Plate 4	FAM (*p102*)	100	0.998	-3.322	40.2
	Cy5 (fruA)	97.5	0.996	-3.382	39.5
	Texas Red (p37)	100.9	0.996	-3.301	39.2
Plate 5	FAM (*p102*)	99.3	0.997	-3.339	38.5
	Cy5 (fruA)	92.3	0.999	-3.522	40.5
	Texas Red (p37)	97.3	0.999	-3.388	38.6
Plate 6	FAM (*p102*)	99.2	0.997	-3.341	38.7
	Cy5 (fruA)	97.8	0.993	-3.375	39.4
	Texas Red (p37)	96.8	0.999	-3.401	38.6

r^2: determination coefficient.

Additional file 3: Detailed information on the individual samples tested by multiplex-qPCR.

		BALF samples						NT samples					
		M. hyopneumoniae		*M. flocculare*		*M. hyorhinis*		*M. hyopneumoniae*		*M. flocculare*		*M. hyorhinis*	
	Sample ID	**Cq mean**	**SQ mean**	**Cq mean**	**SQ mean**	**Cq mean**	**SQ mean**	**Cq mean**	**SQ mean**	**Cq mean**	**SQ mean**	**CQ mean**	**Sq mean**
Herd 1 (H1)	4.1	0.00	0.00E+00	20.24	4.34E+05	*	*	0.00	0.00E+00	25.10	1.79E+04	24.52	1.36E+04
	4.2	0.00	0.00E+00	18.88	1.13E+06	0.00	0.00E+00	0.00	0.00E+00	25.14	1.73E+04	27.95	1.34E+03
	4.3	0.00	0.00E+00	22.28	1.04E+05	0.00	0.00E+00	0.00	0.00E+00	25.01	1.92E+04	35.18	1.55E+01
	4.4	0.00	0.00E+00	20.90	2.73E+05	31.95	1.11E+02	0.00	0.00E+00	25.96	1.01E+04	25.19	8.70E+03
	4.5	0.00	0.00E+00	21.92	1.34E+05	29.03	7.83E+02	0.00	0.00E+00	24.73	2.32E+04	30.63	4.28E+02
	4.6	0.00	0.00E+00	26.29	6.28E+03	32.78	5.76E+01	0.00	0.00E+00	28.50	1.80E+03	28.48	9.36E+02
	4.7	0.00	0.00E+00	26.48	5.51E+03	0.00	0.00E+00	0.00	0.00E+00	28.00	2.47E+03	0.00	0.00E+00
	4.8	0.00	0.00E+00	0.00	0.00E+00	24.60	1.72E+04	0.00	0.00E+00	27.58	3.29E+03	29.39	5.20E+02
	4.9	0.00	0.00E+00	23.23	5.34E+04	*	*	0.00	0.00E+00	27.41	3.69E+03	28.36	1.03E+03
	4.10	0.00	0.00E+00	22.08	1.20E+05	31.03	1.94E+02	0.00	0.00E+00	25.66	1.21E+04	31.62	1.22E+02
	4.11	0.00	0.00E+00	23.35	4.93E+04	*	*	0.00	0.00E+00	23.69	4.78E+04	33.03	4.67E+01
	4.12	0.00	0.00E+00	21.39	1.94E+05	28.19	1.40E+03	0.00	0.00E+00	24.96	1.97E+04	29.89	3.59E+02
	4.13	0.00	0.00E+00	26.45	5.62E+03	26.69	4.01E+03	0.00	0.00E+00	26.66	6.21E+03	29.61	4.36E+02
	4.14	0.00	0.00E+00	20.14	4.68E+05	31.01	2.22E+02	0.00	0.00E+00	24.14	3.45E+04	27.20	2.23E+03
	4.15	0.00	0.00E+00	26.88	4.19E+03	0.00	0.00E+00	0.00	0.00E+00	26.53	6.73E+03	0.00	0.00E+00
	4.16	0.00	0.00E+00	23.16	6.94E+04	0.00	0.00E+00	0.00	0.00E+00	26.39	7.63E+03	26.94	2.67E+03
	4.17	0.00	0.00E+00	21.25	2.54E+05	0.00	0.00E+00	0.00	0.00E+00	26.82	5.68E+03	33.92	2.40E+01
	4.18	0.00	0.00E+00	21.39	2.31E+05	0.00	0.00E+00	0.00	0.00E+00	25.26	1.59E+04	28.01	1.30E+03
	4.19	0.00	0.00E+00	23.53	5.40E+04	0.00	0.00E+00	0.00	0.00E+00	25.78	1.12E+04	25.02	9.73E+03
	4.20	0.00	0.00E+00	21.24	2.56E+05	28.60	1.61E+03	0.00	0.00E+00	0.00	0.00E+00	23.94	2.03E+04
	4.21	0.00	0.00E+00	23.18	6.85E+04	0.00	0.00E+00	0.00	0.00E+00	0.00	0.00E+00	25.89	5.41E+03
	4.22	0.00	0.00E+00	17.99	2.34E+06	36.23	7.85E+00	0.00	0.00E+00	0.00	0.00E+00	24.16	1.74E+04
Herd 2 (H2)	1.1	22.08	2.47E+05	25.61	9.96E+03	0.00	0.00E+00	0.00	0.00E+00	0.00	0.00E+00	28.51	9.87E+02
	1.2	19.68	1.27E+06	22.15	1.04E+05	27.89	9.27E+02	32.00	1.62E+02	31.07	4.82E+02	0.00	0.00E+00
	1.3	0.00	0.00E+00	24.24	2.53E+04	22.80	3.19E+04	28.04	1.41E+03	0.00	0.00E+00	24.69	1.30E+04
	1.4	19.95	1.06E+06	28.31	1.59E+03	*	*	0.00	0.00E+00	0.00	0.00E+00	30.34	2.86E+02
	1.5	18.26	3.36E+06	21.99	1.15E+05	0.00	0.00E+00	*	*	22.36	1.43E+05	33.97	2.30E+01
	1.6	17.42	9.94E+06	20.36	3.34E+05	*	*	0.00	0.00E+00	24.85	2.80E+04	0.00	0.00E+00
	1.7	24.10	9.40E+04	28.18	1.53E+03	0.00	0.00E+00	0.00	0.00E+00	30.02	9.51E+02	0.00	0.00E+00
	1.8	16.18	2.36E+07	0.00	0.00E+00	0.00	0.00E+00	0.00	0.00E+00	25.59	1.76E+04	30.71	2.17E+02
	1.9	19.50	2.33E+06	23.08	5.08E+04	35.61	1.01E+01	35.75	1.18E+01	*	*	24.45	1.52E+04
	1.10	17.32	1.07E+07	24.24	2.38E+04	27.86	2.28E+03	0.00	0.00E+00	27.26	5.76E+03	33.46	6.00E+01

	1.11	19.69	2.05E+06	22.97	5.50E+04	33.01	6.28E+01	*	*	27.03	4.80E+03	30.07	3.43E+02
	1.12	17.15	1.20E+07	0.00	0.00E+00	0.00	0.00E+00	0.00	0.00E+00	25.06	2.43E+04	31.18	1.58E+02
	1.13	15.25	4.51E+07	38.99	1.07E+00	0.00	0.00E+00	26.75	3.37E+03	25.03	2.46E+04	*	*
	1.14	15.37	4.18E+07	18.19	1.49E+06	24.46	2.50E+04	0.00	0.00E+00	0.00	0.00E+00	22.57	5.48E+04
	1.15	17.70	8.20E+06	30.08	4.16E+02	33.58	4.21E+01	0.00	0.00E+00	30.49	4.56E+02	0.00	0.00E+00
	1.16	15.67	3.37E+07	12.54	7.31E+07	17.65	2.90E+06	0.00	0.00E+00	24.94	2.68E+04	0.00	0.00E+00
	1.17	21.87	4.45E+05	39.82	4.97E-01	32.76	7.46E+01	0.00	0.00E+00	25.75	1.60E+04	33.66	2.96E+01
	1.18	17.67	8.37E+06	23.15	4.87E+04	0.00	0.00E+00	27.30	2.32E+03	29.88	8.68E+02	0.00	0.00E+00
	1.19	16.00	2.69E+07	29.19	1.12E+03	27.85	2.32E+03	0.00	0.00E+00	0.00	0.00E+00	29.96	3.77E+02
	1,20	23.96	1.03E+05	25.08	1.29E+04	32.67	7.95E+01	26.43	4.20E+03	0.00	0.00E+00	33.73	2.87E+01
	1.21	21.37	6.30E+05	26.85	4.54E+03	32.01	1.26E+02	0.00	0.00E+00	26.17	1.17E+04	31.51	1.21E+02
	1.22	21.82	4.61E+05	28.54	1.18E+03	0.00	0.00E+00	0.00	0.00E+00	0.00	0.00E+00	28.68	8.78E+02
	3.1	31.21	6.59E+02	29.85	4.98E+02	0.00	0.00E+00	0.00	0.00E+00	25.57	1.74E+04	0.00	0.00E+00
	3.2	32.34	2.98E+02	26.10	6.39E+03	28.45	1.51E+03	0.00	0.00E+00	24.37	3.80E+04	0.00	0.00E+00
	3.3	21.25	6.88E+05	26.22	5.85E+03	*	*	0.00	0.00E+00	29.30	1.56E+03	0.00	0.00E+00
	3.4	24.02	9.92E+04	0.00	0.00E+00	0.00	0.00E+00	0.00	0.00E+00	31.05	4.89E+02	34.34	1.87E+01
	3.5	27.25	1.04E+04	*	*	34.31	2.51E+01	0.00	0.00E+00	30.15	8.90E+02	0.00	0.00E+00
	3.6	23.39	1.54E+05	28.08	1.63E+03	0.00	0.00E+00	0.00	0.00E+00	30.69	6.30E+02	0.00	0.00E+00
	3.7	26.81	1.42E+04	28.83	9.67E+02	0.00	0.00E+00	0.00	0.00E+00	0.00	0.00E+00	0.00	0.00E+00
	3.8	18.60	4.39E+06	0.00	0.00E+00	*	*	0.00	0.00E+00	29.88	1.06E+03	0.00	0.00E+00
	3.9	20.18	1.45E+06	0.00	0.00E+00	0.00	0.00E+00	0.00	0.00E+00	26.75	8.02E+03	0.00	0.00E+00
	3.10	23.79	1.16E+05	26.24	5.89E+03	0.00	0.00E+00	0.00	0.00E+00	28.36	1.94E+03	0.00	0.00E+00
Herd 3 (H3)	3.11	26.66	1.58E+04	26.87	4.18E+03	0.00	0.00E+00	0.00	0.00E+00	29.45	1.40E+03	0.00	0.00E+00
	3.12	24.00	9.69E+04	0.00	0.00E+00	0.00	0.00E+00	0.00	0.00E+00	26.23	1.14E+04	0.00	0.00E+00
	3.13	27.07	1.19E+04	0.00	0.00E+00	0.00	0.00E+00	0.00	0.00E+00	0.00	0.00E+00	0.00	0.00E+00
	3.14	20.33	1.19E+06	29.84	5.34E+02	0.00	0.00E+00	29.60	4.73E+02	30.78	3.91E+02	0.00	0.00E+00
	3.15	19.57	1.99E+06	0.00	0.00E+00	0.00	0.00E+00	27.57	1.91E+03	24.97	2.58E+04	0.00	0.00E+00
	3.16	22.78	2.23E+05	0.00	0.00E+00	0.00	0.00E+00	0.00	0.00E+00	28.30	3.00E+03	0.00	0.00E+00
	3.17	19.11	2.72E+06	0.00	0.00E+00	0.00	0.00E+00	30.00	3.67E+02	29.43	1.40E+03	0.00	0.00E+00
	3.18	22.93	2.01E+05	31.78	1.35E+02	33.11	4.67E+01	0.00	0.00E+00	29.39	9.54E+02	*	*
	3.19	18.47	4.20E+06	0.00	0.00E+00	35.15	1.11E+01	0.00	0.00E+00	28.23	2.11E+03	0.00	0.00E+00
	3.20	*	*	24.78	1.81E+04	28.84	8.93E+02	0.00	0.00E+00	27.96	2.54E+03	0.00	0.00E+00
	3.21	22.12	3.50E+05	34.86	2.45E+01	0.00	0.00E+00	0.00	0.00E+00	25.81	1.09E+04	0.00	0.00E+00
	3.22	16.67	1.43E+07	0.00	0.00E+00	27.99	1.61E+03	35.56	9.51E+00	33.56	5.52E+01	35.43	9.83E+00

*Cq and SQ ranges could not be defined due to the Monte Carlo effect.

Cq = quantification cycle value; SQ = starting quantity (copies/µL)

Chapter 4 | Rationalizing the use of common parameters and technological tools to follow up *Mycoplasma hyopneumoniae* infections in pigs

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Abstract

Mycoplasma (M.) hyopneumoniae is associated with respiratory disease in pigs and is the primary agent of enzootic pneumonia. Quantification of *M. hyopneumoniae*-related outcome parameters can be difficult, expensive, and time-consuming, in both research and field settings. In addition to well-established methods, technological tools are becoming available to monitor various aspects of relevant animal- and environment-related features, often in real-time. Therefore, this study aimed to assess whether certain parameters, such as animal movement and body temperature using microchips (IMT), correlate with established parameters and whether the currently used parameters can be rationalized. The percentage of movement was significantly reduced by *M. hyopneumoniae* infection in pigs ($p < 0.05$), where the *M. hyopneumoniae*-infected group showed a lower percentage of movement (1.9%) when compared to the negative control group (6.9%). On the other hand, macroscopic (MLCL) and microscopic (MLL) lung lesions, respiratory disease score (RDS), *M. hyopneumoniae*-DNA load, and anti-*M. hyopneumoniae* antibody levels increased significantly in the *M. hyopneumoniae*-infected group 28 days post-inoculation ($p < 0.05$). Moderate ($r > 0.30$) to very strong correlations (> 0.80) were observed between the abovementioned parameters ($p < 0.05$), except for IMT. A significant and moderate correlation was reported between IMT and rectal temperature ($r= 0.49$; $p < 0.05$). Last, the average daily weight gain and the percentage of air in the lung were not affected by *M. hyopneumoniae* infection ($p > 0.05$). *M. hyopneumoniae* infection significantly reduced the movement of piglets and increased lung lesions, bacterial load, and antibody levels; and, good correlations were observed between all parameters, indicating a direct relationship between them. Thus, we suggest that changes in movement can be a reliable indicator of *M. hyopneumoniae* infection in pigs, and that a selected group of parameters—specifically RDS, MLCL, MLL, *M. hyopneumoniae*-DNA load, anti-*M. hyopneumoniae* antibodies, and movement—are optimal to assess *M. hyopneumoniae* infection under experimental conditions.

Keywords: movement, microchip, sensors, respiratory disease, experimental infection, swine

Background

Respiratory diseases are a major health challenge in swine production worldwide. This is especially the case for enzootic pneumonia (EP), a chronic respiratory disease, that negatively impacts the health and growth of pigs. *Mycoplasma hyopneumoniae (M. hyopneumoniae)* is the primary pathogen of EP and plays an important role within the Porcine Respiratory Disease Complex (PRDC), together with other pathogens such as Porcine circovirus 2, porcine respiratory reproductive syndrome virus (PRRSV), and *Pasteurella multocida* (Maes et al., 2023).

M. hyopneumoniae colonizes the mucosal surface of the trachea, bronchi, and bronchioles, affecting the mucosal clearance system by breaking the cilia and modulating the respiratory tract immune system (Maes et al., 2018b; Pieters and Maes, 2019). Strains of *M. hyopneumoniae* have been described to be antigenically and genetically diverse (Assao et al., 2019; Betlach et al., 2019; Charlebois et al., 2014; Pieters and Maes, 2019), and differences in virulence have been reported, with high virulence strains inducing more severe pneumonia in a greater proportion of infected pigs (Abt et al., 2012; Meyns et al., 2007; Meyns et al., 2004; Vicca et al., 2003a; Woolley et al., 2012). This might be attributed to a greater capacity of the bacteria to multiply in the bronchioles, resulting in a more severe inflammation (Meyns et al., 2007). Additionally, the pathogenesis of *M. hyopneumoniae* is complex and involves long-term colonization of the ciliated epithelium, stimulation of a prolonged inflammatory reaction, modulation of innate and adaptive immune responses, and interaction with other infectious agents (Pieters and Maes, 2019).

Although an acute inflammatory response is triggered by *M. hyopneumoniae* infection in the lungs, the disease predominantly occurs as a chronic condition, in which the unproductive cough is the most prominent clinical sign (Pieters and Maes, 2019; Vicca et al., 2003b). In addition to that, shortness of breath, dyspnea, and fever may occur upon infection. Macroscopic lesions in the lungs can be found in the apical, cardiac, and accessory lobes and, sometimes, in the cranioventral part of the diaphragmatic lobes (Pieters and Maes, 2019). These lesions consist of consolidated areas that range from purple to gray (Garcia-Morante et al., 2016). At the microscopic level, *M. hyopneumoniae* triggers the development of broncho-interstitial pneumonia, initiating with limited

neutrophil accumulation and progressing to marked accumulation of neutrophils, fluid, and macrophages in alveoli. This leads to the proliferation of type II alveolar pneumocytes, increased lymphocyte, histiocyte, and plasma cell accumulation, peribronchial lymphoid nodules, and subsequent development of alveolar pneumonia. The final stages involve peribronchial lymphoid hyperplasia, increased perivascular mononuclear cells, and extensive peribronchial cuffs with numerous lymphocytes and fewer plasma cells, forming large compressive lymphoid nodules (Blanchard et al., 1992; Morris et al., 1995a; Pieters and Maes, 2019).

Diagnostics of *M. hyopneumoniae* infection comprehend the evaluation of non-pathognomonic parameters, such as clinical signs (cough, fever), macroscopic and microscopic lung lesions, average daily weight gain, percentage of air in the lung, and specific diagnostic tools for direct or indirect detection of *M. hyopneumoniae*, for example using PCR (Beuckelaere et al., 2022; Fourour et al., 2018a; Strait et al., 2008) and ELISA tests (Pieters and Maes, 2019). Although commonly used, evaluating these parameters might be labor-intensive, time-consuming, and expensive (Chae et al., 2021), raising questions on the added value of some parameters (Garcia-Morante et al., 2022). Furthermore, the meticulous selection of the most relevant parameters to be evaluated in *M. hyopneumoniae*-related research would enable us to optimize diagnostic accuracy significantly, thereby maximizing the efficiency of the allocated resources (Garcia-Morante et al., 2022; Thacker, 2004).

Since there is an increasing need for real-time monitoring of health and performance parameters in pig farming to increase productivity and sustainability, recent technologies like intelligent sensors (cameras, microphones, and microchips) and artificial intelligence are being introduced (Wang et al., 2022). Moreover, these technologies can be easily applied in experimental settings to optimize challenge models, efficacy studies, and clinical diagnosis (Pieters and Maes, 2019; Wang et al., 2022). An interesting parameter to monitor is the movement of animals, as changes in the frequency of movement have been shown to be a key indicator of health or welfare problems and to assist in early disease detection (Fernandez-Carrion et al., 2017). In addition, in early infections, one of the first physiological changes is often the increase in body temperature,

and thus, being able to assess body temperature without placing the pigs under unnecessary stress e.g., by using thermal microchips or infrared cameras, can be valuable (Arulmozhi et al., 2021; Burdick Sanchez et al., 2023). Although the movement behavior of pigs has been assessed for some respiratory diseases, such as Porcine Reproductive and Respiratory Syndrome (PRRS) and porcine pleuropneumonia (*Actinobacillus pleuropneumoniae*), it has not yet been investigated for *M. hyopneumoniae* infection. Likewise, changes in intramuscular temperature (IMT) have not yet been monitored during *M. hyopneumoniae* infection. Therefore, the aims of the present study were: i) to assess the impact of *M. hyopneumoniae* infection on the movement and the IMT of piglets by using a camera monitor and a thermal microchip; ii) to investigate the correlations between movement, IMT, and the conventionally used parameters, such as respiratory disease score, macroscopic and microscopic lung lesions, percentage of air, average daily weight gain, bacterial load, and antibody levels.

Methods

Experimental design

This study was approved by the Ethics Committee of the Faculty of Veterinary Medicine and the Faculty of Bioscience Engineering at Ghent University (EC 2021-062) and complies with European Directive 2010/63/EU.

Thirty-two, 28-day-old piglets (16 males and 16 females; Naima x Piétrain), were purchased from a *M. hyopneumoniae*-free herd, which is a farrow to finish farm with 300 sows and producing according to a three-week system. The herd has previously tested seronegative and PCR-negative for *M. hyopneumoniae* (Beuckelaere et al., 2022), and no *Mycoplasma hyopneumoniae*-like lung lesions were reported during the last four years. One month before the start of the trial, tracheobronchial swabs were collected from 50 pigs of five different age groups (7-8 weeks, 11 weeks, 16 weeks, 22 weeks, and 26 weeks) and tested by nPCR for the presence of *M. hyopneumoniae* DNA (Stark et al., 1998). All samples tested negative. The farm was also free from PRRSV and *Actinobacillus pleuropneumoniae.*

The piglets were transported to the research facilities of the Faculty of Veterinary Medicine, at Ghent University, when they were 28 days of age (D-12). Upon arrival, the piglets were weighted, identified with numbered ear tags, and microchips (2.12 x 13 mm) with Bio-Thermo technology were implanted intramuscularly (LifeChip®; Allflex, Belgium) for temperature measurement. Then, the animals were randomly allocated to four different groups (n=8/group) (Figure 1), and able to acclimatize for 12 days.

Each group was housed in one of the four separate, but similar HEPA filtered compartments, and with free access to drinking water and feed. The animals were fed with a commercial feed free from antibiotics. In addition, feed and water management, environmental conditions, and other factors such as flooring, heating, the number and location of toys, and feeders were consistent among the different groups. Each compartment was equipped with a Healthy Climate Monitor (HCM; Healthy Climate Solutions, The Netherlands), a device used to monitor the movement, sound levels, and environmental parameters in pig farms. Details about the HCM are described under the section “Assessment of movement, sound, and environmental conditions”.

If pigs showed clinical signs of post-weaning diarrhea, they were treated orally with colistin (Colivet® quick-pump, Prodivet, Belgium), and in case of lameness, they were treated intramuscularly with amoxicillin (Duphamox LA, Zoetis, Belgium) and meloxicam (Melovem®, Dopharma, Belgium), following the leaflet dosage.

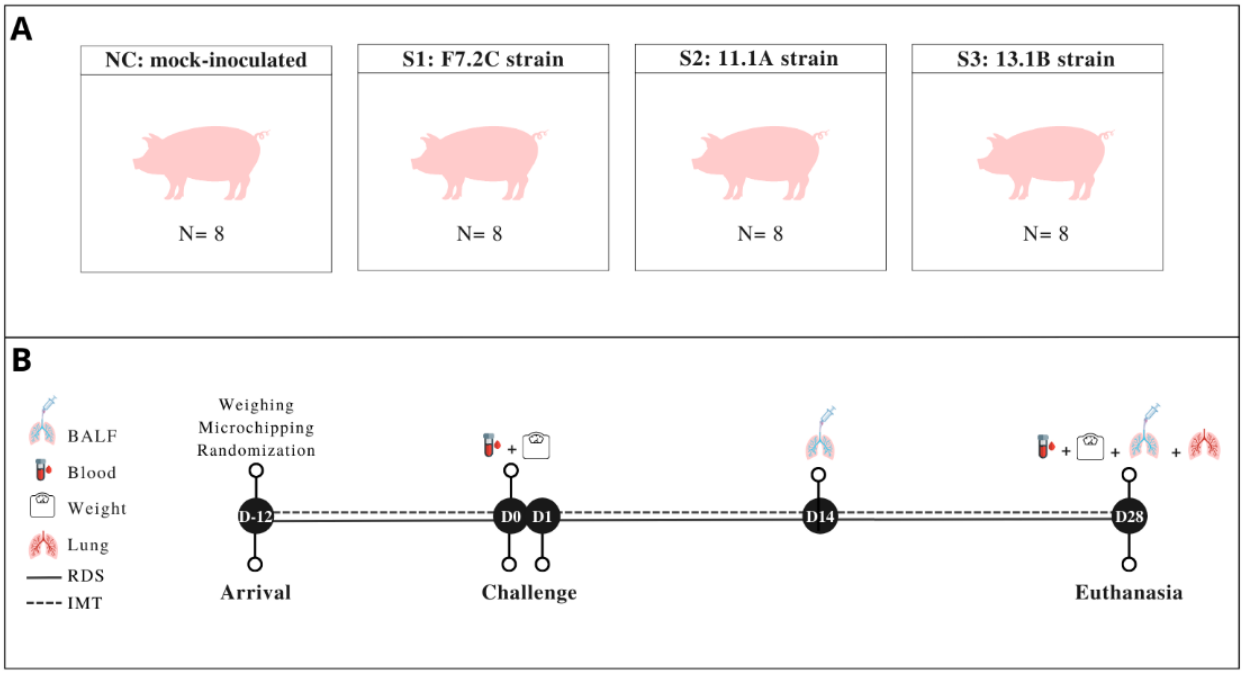


Figure 1: Schematic overview of the study design. A) Thirty-two *M. hyopneumoniae*-free piglets were randomly allocated to the four groups. B) Overview of the timeline and sampling events. NC: negative control group inoculated with sterile medium on D0 and D1; S1: group infected with the *M. hyopneumoniae* strain F7.2C (D0) and F1.12A (D1); S2: group infected with the recently isolated *M. hyopneumoniae* strain 11.1A (D0) and F1.12A (D1); S3: group infected with the recently isolated *M. hyopneumoniae* strain 13.1B (D0) and F1.12A (D1); BALF: broncho-alveolar lavage fluid; RDS: respiratory disease score; IMT: intramuscular temperature, D: day.

Isolates

The F7.2C strain (GenBank accession #NC_007295) and F1.12A strain (GenBank accession #KY264124.1) were isolated in the year 2000 and were characterized as highly and low virulent strains (Vicca et al., 2003a), respectively. On the other hand, the strains 11.1A (GenBank accession # CP098241) and 13.1B (GenBank accession # CP098240) were recently isolated (2021) from pigs with *Mycoplasma*-like lung lesions at slaughter. Their virulence has not yet been studied.

The new strains were isolated from two different fattening herds in Belgium, and whole-genome sequencing (WGS), on a MinION flow cell (Oxford Nanopore Technologies), confirmed that they belong to the *M. hyopneumoniae* species and were distinctly related to the F7.2C strain. The pairwise identity ranged from 97.92 to 99.45 % (BLASTN, v2.10.11; data not published). Last, the genome of the isolates 11.1A and 13.1B is 0.95Mbp and 0.98Mbp, respectively (data not published).

Inoculum preparation and challenge

One milliliter of each *M. hyopneumoniae* isolate (13.1B, 11.1A, F7.2C, and F1.12A) was inoculated in a flask (one for each strain) containing 400 ml of Friis medium (Calus et al., 2010; Friis, 1975), which was maintained in an incubator at 37 °C until ATP values (Calus et al., 2010) were greater than 430 pmol ATP/ml. The determination of the concentration of *M. hyopneumoniae* was carried out by successive dilutions of the culture in Friis medium as described elsewhere (Calus et al., 2010). Then, the inoculum was aliquoted into 50 ml sterile falcons and stored at -80 °C until use. Sterility tests were performed for all flasks on blood agar and McConkey media, and left in an incubator at 37 °C for two days. The sterility test turned out to be negative for all cultures/flasks.

All pigs were inoculated at 40 and 41 days of age (D0 and D1). On the first inoculation day (D0), piglets (n = 8) in each infected group received one of the three different *M. hyopneumoniae* strains namely F7.2C (S1), 11.1A (S2), or 13.1B (S3). On the following day (D1), piglets in the three infected groups were additionally inoculated with a low virulent strain (F1.12A). The negative control group was inoculated with sterile Friis medium at both time points (D0 and D1).

Before inoculation, animals were anesthetized with 0.22 mL/kg body weight of a mixture of Zoletil 100® (Virbac, Belgium) and Xyl-M® 2% (VMD, Belgium), which was given intramuscularly according to label instructions. The challenge was performed by endotracheal inoculation of 7 ml of each *M. hyopneumoniae* strain containing 10^8 CCU/ml (Calus et al., 2010) or sterile Friis medium.

Assessment of movement, sound, and environmental conditions

The HCM (Healthy Climate Solutions, The Netherlands) is a tool designed for continuous monitoring of indoor farm climate. The device measures room temperature, relative humidity, carbon dioxide (CO_2), ammonia (NH_3), dust particles (10 µg/m^3 and 1 µg/m^3), and air pressure. In addition, it captures high-definition photos to assess movement and includes an optional microphone to assess noise levels (602 Hz). The device transmits its data to the Healthy Climate Monitor app once per minute over a 4G connection, which can then be assessed by the researcher. The percentage of movement was calculated automatically by an algorithm (Healthy Climate Solutions Api, version 2.7.3), which compares the changes in pixels between images (every 5 minutes), with a resolution of 1640x1232px.

In each room, an HCM device was installed on the ceiling in the middle of the room, away from the air inlet, and approximately two meters high from the floor, allowing a complete view over the pen and the animals (Additional file 10). The movement of the piglets was continuously measured by the HCM from D-11 to D28. However, only measurements from 11 am to 5 pm were used to avoid possible interference with the handling of the animals in the morning, and also to avoid bias from technical issues during the nighttime (like not capturing pictures or sudden changes in brightness), which interfered with the pixels count, and therefore the percentage of movement. Lastly, the percentage of movement was calculated as the daily mean of the measurements taken within a 24-hour period.

Assessment of clinical signs, body temperature, and average daily weight gain

During the study, the animals were observed daily, by the same person, for at least 20 minutes in the morning (between 8-10am). The severity of coughing was measured using a Respiratory Disease Score (RDS) (Halbur et al., 1996), which ranges from 0 to 6, where: 0 = no coughing, 1 = mild coughing after an encouraged move, 2 = mild coughing in rest, 3 = moderate coughing after an encouraged move, 4 = moderate coughing in rest, 5 severe coughing after an encouraged move, 6 = severe coughing in rest. Other possible

clinical findings, such as loss of appetite, diarrhea, dyspnea, and lameness were also recorded.

Two different methods were used to evaluate the body temperature of the piglets: manually taken rectal temperature (RT) and with a transponder-read thermal microchip (LifeChip®, Allflex, Belgium). The microchip was implanted intramuscularly upon arrival (D-12), on the right side of the neck (Additional file 11), according to the instructions provided by the manufacturer. The individual intramuscular temperature was assessed daily in the morning (between 8.00 and 10.00) from D-12 to D28 by reading the transponder with a portable stick reader RS 420 (Allflex, Belgium). The RT was measured daily from D-7 to D-3 (between 8.00 and 10.00), and after the intramuscular temperature (IMT) measurement, with a calibrated thermometer. Last, to investigate possible migration, the location of the microchips was checked in 20% of the animals at euthanasia.

All piglets were weighed upon arrival (D-12), before inoculation (D0), and at euthanasia (D28) to evaluate the average daily weight gain (ADWG). The ADWG was calculated by subtracting the weight at D-12 or D0 from the final weight (D28) and dividing the difference by the number of days between the time points.

Blood and broncho-alveolar lavage fluid (BALF) sampling

Blood samples were collected just before inoculation (D0) and at euthanasia (D28) by puncturing the jugular vein, using sterile disposable needles and vacutainer tubes with clot activator (Vacutainer - BD, USA). Blood samples were centrifuged at 1500x*g* for 10 minutes and the serum was aliquoted into sterile microtubes and stored at -20 °C until use.

The BALF samples were collected two weeks after inoculation (D14) and at euthanasia (D28). At D14, the animals were restrained using a snare and a catheter (Portex® Dog Catheter with Female Luer Mount, United Kingdom) was inserted into the trachea. Then, 20 mL sterile PBS was flushed into the lung and immediately aspirated back. At D28, BALF samples were collected after euthanasia by flushing the head bronchus of the right lung with 20 mL sterile PBS, followed by immediate aspiration of the

fluid. All BALF samples were aliquoted into sterile microtubes and stored at -80 °C until further testing by PCR.

Macroscopic and microscopic lung lesion evaluation

After the lungs were removed from the cadaver at necropsy (D28), the EP-like lung lesion or macroscopic lung consolidated lesions (MLCL) were scored according to Hannan et al. (Hannan et al., 1982). The score ranges from 0 (no EP-like lesions) to 35 (entire lung affected by EP-like lesions).

For the microscopic lung lesion (MLL) evaluation, samples from the left lung (apical, cardiac, and diaphragmatic lobes) were collected (Hannan et al., 1982), fixed in 10% neutral buffered formalin for 48 hours, and processed for hematoxylin and eosin staining. If *Mycoplasma*-like lesions were present, the sample was collected from the transition area of the lesion. The microscopic slides were digitalized using a Nanozoomer NDP slide scanner (Hamamatsu Photonics, Japan) and its viewing platform (NDP.View2). To get the average lesion score, the degree of peribronchiolar and perivascular lymphohistiocytic infiltration and nodule formation (cuffing) was assessed on 10 microscopic fields (10x magnification) using a previously described scoring system (Morris et al., 1995b; Vicca et al., 2003a). The score ranges from 1 – 5, as described below and exemplified in Additional file 12.

Table 5: Classification of the microscopic findings as described by Morris et al. (1995b)

Score	Description
1	Limited infiltration of macrophages and lymphocytes around bronchioles, with airways and alveolar spaces free of cellular exudates.
2	Light to moderate infiltrates with mild diffuse cellular exudates into airways.
3	Mild lesions characteristic of broncho-interstitial pneumonia, centered around bronchioles but extending to the interstitium, with lymph follicular infiltration and mixed inflammatory cell exudates.
4	Moderate lesions characteristic of broncho-interstitial pneumonia, centered around bronchioles but extending to the interstitium, with lymph follicular infiltration and mixed inflammatory cell exudates.
5	Severe lesions characteristic of broncho-interstitial pneumonia, centered around bronchioles but extending to the interstitium, with lymph follicular infiltration and mixed inflammatory cell exudates.

The percentage of lung area occupied by air (% of air) in the lung was determined by analyzing the same 10 microscopic fields per lobe with the ImageJ program (Bethesda Softworks, USA), as reported elsewhere (Beuckelaere et al., 2022; Michiels et al., 2017a).

Routine bacteriological culture

Ten microliters of each BALF sample collected at euthanasia (D28) were inoculated on Columbia blood agar plates with 5% sheep blood (Oxoid, United Kingdom) with a *Staphylococcus pseudintermedius* streak, as reported previously (Villarreal et al., 2011). All plates were incubated for 48 h at 37°C in a 5 % CO_2-enriched environment to isolate other bacteria, especially respiratory pathogens. Then, the different colonies were identified at the species level (score value > 2.000) using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF; Bruker Daltonics, Germany) as previously described (Beuckelaere et al., 2022; Bizzini and Greub, 2010; Clark et al., 2013).

Anti-M. hyopneumoniae antibody detection

To detect serum antibodies at D0 and D28, the commercial kit *M.hyo Ab test* (Idexx, USA) was used, following the manufacturer's instructions. Absorbance values were read in a spectrophotometer at 650 nm wavelength. All samples that presented a S/P value higher than 0.3 were considered positive for anti-*M. hyopneumoniae* antibodies (Almeida et al., 2020c).

DNA extraction and digital PCR (dPCR) for M. hyopneumoniae

A total of 200 µL of each BALF sample was used for DNA extraction with the DNeasy Blood & Tissue kit (QIAGEN, Belgium), according to the manufacturer's protocol. Then, digital droplet PCR (dPCR) was performed as described previously (Beuckelaere et al., 2022). The assay is based on the primers and probe described by Marois et al. (2010), which target the *p*102 adhesin gene (Table 6).

Table 6. Primers and probe sequences used for amplifying the *p*102 gene of *M. hyopneumoniae*.

ID	Sequence (5'-3')
Forward	GTCAAAGTCAAAGTCAGCAAAC
Reverse	AGCTGTTCAAATGCTTGTCC
Probe	Cy5-ACCAGTTTCTAOCACTTCATCGCCTCA-IAbRQSp

The assay was performed with the droplet-based Naica System (Stilla Technologies, France) using 16 reaction cavity Naica Opal chips (Stilla Technologies, France). Briefly, the dPCR mix (8 µL) containing 1x PerfeCTa® Multiplex qPCR Toughmix® (VWR International BVBA, Belgium), 0.25 µmol/L of the probe, 0.5 µmol/L of each primer, 0.1 µmol/L fluorescein (Merck, Germany), 0.4 µL Fast Digest EcoRi (Thermo Fisher Scientific, USA) and 1.6 µL sample DNA. Highly concentrated samples were diluted using the UltraPure™ Salmon Sperm DNA Solution (10 µg/mL, Thermo Fisher Scientific, USA). For each run, at least two non-template controls (NTC) were included. The thermal cycling

was performed on the Geode device (Stilla Technologies, France), where initial denaturation (10 minutes at 95 °C) was followed by 40 amplification cycles of denaturation for 15 seconds at 95 °C and annealing/elongation for 30 seconds at 60 °C. Fluorescence reading was done using the Naica Prism3 System (Stilla Technologies, France). The threshold value was based on the NTC samples, as described elsewhere (Beuckelaere et al., 2022).

Statistical analyses

The results were analyzed using IBM SPSS® Statistics Version 28 (IBM, Chicago, USA). For all models, the assumption of normality was tested by examining histograms, and the homogeneity of variance of the final models was tested by examining histograms and the QQ plot of the residuals. The assumption of homogeneity of residuals was checked before using parametric tests/models (*e.g.* Bland Altman test) by the Shapiro-Wilk test. For the mixed models used in this study, the assumption of homogeneity of residuals was checked by plotting residuals versus fitted values.

To evaluate the data statistically, all animals in the infected groups (S1, S2, and S3) were pooled and considered as one group, the *M. hyopneumoniae*-infected (*M. hyopneumoniae*) group, while the NC remained as the negative control group. The linear mixed model with repeated measurements was used to evaluate the movement data, in which movement was the dependent variable and infection status was the independent variable. The Generalized Estimating Equations model was used to analyze the RDS data, where the infected groups were pooled and considered as one group (independent variable).

The correlations between the main clinical parameters (MLCL, MLL, IMT, RT, RDS, *M. hyopneumoniae*-DNA load, anti-*M. hyopneumoniae* antibodies, and percentage of air) were evaluated by the Spearman's method. The association between intramuscular temperature and rectal temperature was assessed by means of Bland Altman graphs and non-parametric tests. Statistical results were considered significant when $p \leq 0.05$.

Results

Six animals (two from each infected group) died during or shortly after the inoculation on D0 or D1, and therefore, they were excluded from all analyses. No specific lesions were observed during the necropsy, indicating that the death of those piglets was probably associated with the stress of the manipulation or other factors intrinsic to the individuals. At euthanasia, mild pleurisy and/or small abscesses were observed in 10 piglets, in which five were from the negative control (NC) group, two from S1, and three from S3. Mild clinical signs of post-weaning diarrhea were observed in all groups from D-6 onwards and were resolved by D-1, after individual treatment with colistin.

In the BALF (D28) of two animals (from NC and S3) *Trueperella pyogenes* and *Pasteurella multocida* were identified. All animals from the control group remained negative for *M. hyopneumoniae*-specific antibodies and DNA throughout the experimental period.

Assessment of movement and environmental conditions

During the post-inoculation period (D0 to D28), the *M. hyopneumoniae*-infected group showed a lower percentage of movement (1.9%) when compared to the negative control group (6.9%; $p < 0.05$) (Figure 2), and the odds of movement after inoculation in the NC group was 28.68 times greater than for the *M. hyopneumoniae* group ($p < 0.001$). Prior to the inoculation, the average percentage of movement was 1.6% for all groups, while upon inoculation with the *M. hyopneumoniae* strains, higher values were observed in the individual infected groups (S1, S2, and S3) when compared to the NC (Additional file 4). Furthermore, the group factor (infected or non-infected) negatively affected the movement ($p < 0.001$), while the factor inoculation (pre- or post-inoculation) showed a tendency to negatively affect the movement ($p = 0.054$).

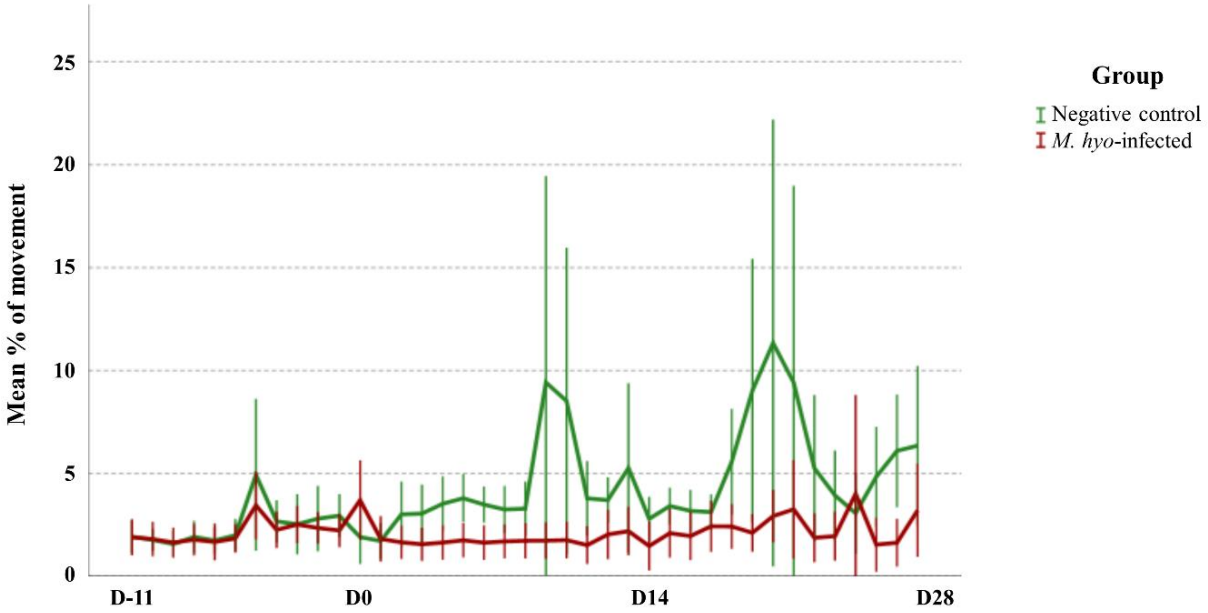


Figure 2: Mean percentage of movement throughout the study period. D0 refers to the inoculation day. Error bars represent the confidence interval of 95%.

Concerning the environmental parameters, similar results were observed among the groups, except for dust particles, for which higher values were observed in the NC (Additional file 5). Similarly, higher decibel values for the 602 Hz frequency were observed in the control group compared to the infected groups (Additional file 5 and 6).

Evaluation of clinical signs and weight gain

Concerning the RDS data, coughing was first reported on D3 in the *M. hyopneumoniae* group and increased progressively until D28, as shown in Additional file 7A. Similarly, a progressive increase in coughing was observed for the individual groups, except for the NC group (Additional file 7B). The RDS post-inoculation was significantly higher in the *M. hyopneumoniae*-infected groups compared to the NC group ($p < 0.05$). Lastly, there were no significant differences in the ADWG between the groups (Table 7).

Table 7. Mean (M) and standard deviation (SD) values of the different parameters assessed in the negative control group and *M. hyopneumoniae*-infected group. Within the same row, values with different letters are significantly different *($p \leq 0.05$)*.

Parameter	Study period	Negative control group		*M. hyopneumoniae*-infected group	
		M	SD	M	SD
RDS post-inoculation	0 to 28	0.06 ^{a}	0.20	0.97 ^{b}	0.83
IMT post-inoculation	0 to 28	39.59 ^{a}	0.20	39.70 ^{a}	0.20
ADWG in g/pig/day	-12 to 0	113.75 ^{a}	48.00	113.56 ^{a}	47.67
	-12 to 28	327.69 ^{a}	90.00	362.11 ^{a}	83.00
	0 to 28	419.38 ^{a}	124.00	468.63 ^{a}	110.00
Anti-M. hyopneumoniae antibodies	28	0.00 ^{a}	0.00	0.67 ^{b}	0.27
M. hyopneumoniae-DNA load (Log10 copies/µL)	14	0.00 ^{a}	0.00	3.85 ^{b}	0.47
	28	0.00 ^{a}	0.00	4.43 ^{b}	0.33
MLCL score	28	0.57 ^{a}	1.30	4.33 ^{b}	2.40
MLL score	28	2.16 ^{a}	0.20	2.63 ^{b}	0.33
% of air in lung slides	28	37.33 ^{a}	4.50	34.56 ^{a}	6.13

RDS: respiratory disease score; IMT: intramuscular temperature (°C); ADWG: average daily weight gain; MLCL: macroscopic lung consolidated lesion; and MLL: microscopic lung lesion.

Assessment of body temperature

Although slightly higher values were reported in the *M. hyopneumoniae*-infected group, we did not see a significant difference in IMT of pigs upon *M. hyopneumoniae*-infection ($p > 0.05$). Likewise, no differences were seen between the individual groups, where values ranged from 38.4 °C to 41.3 °C (Additional file 8B). Variation was 0.21 for the IMT and 0.18 for the RT. A mean difference of 0.25 °C (95% CI = 0.20 – 0.35) was observed ($p < 0.001$) between IMT and RT and in most cases (94/130), IMT was higher, as shown in Figure 3. There was a significant moderate correlation (r=0.53; $p < 0.001$) between IMT and RT (Table 8). Lastly, IMT post-infection did not differ significantly between *M. hyopneumoniae*-infected and NC groups, although the average values for the *M. hyopneumoniae*-infected group were slightly higher (+ 0.11 °C; Additional file 8A).

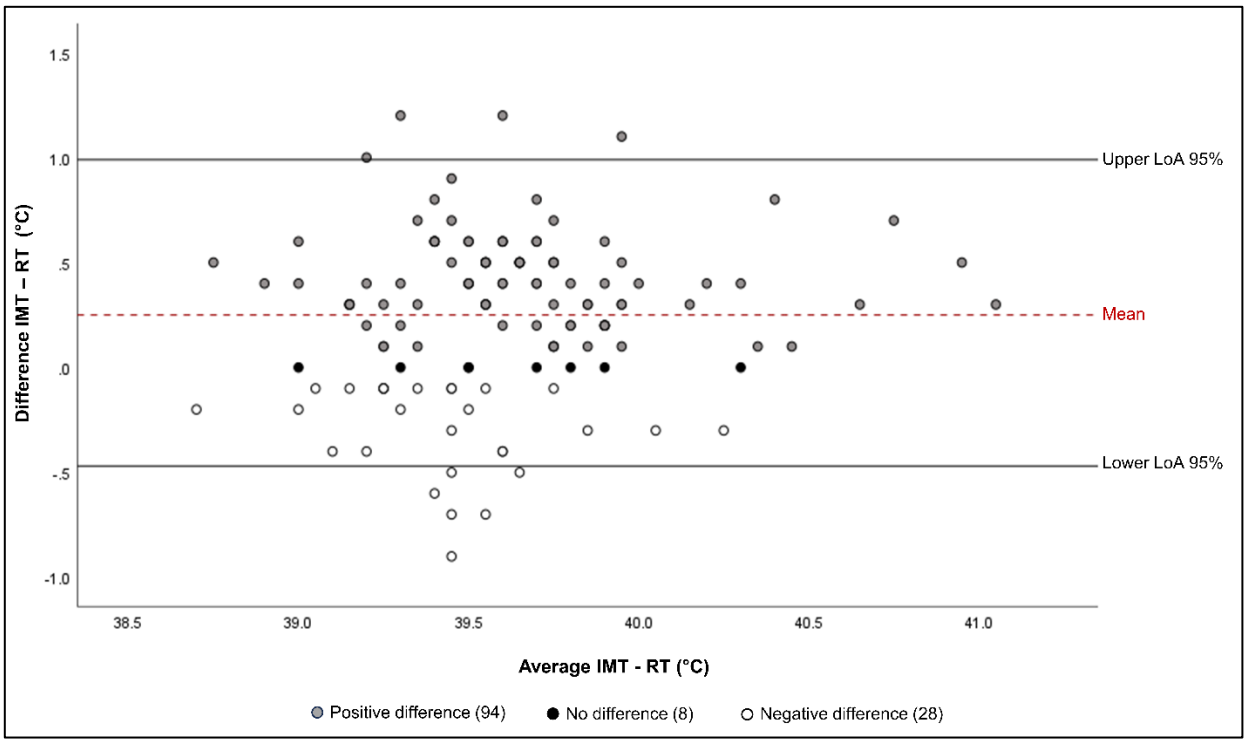


Figure 3: Bland-Altman plot showing the level of agreement (LoA) between intramuscular temperature (IMT) and rectal temperature (RT) measurements, in a 95% confidence interval (CI).

Macroscopic and microscopic lung lesion score

Small *Mycoplasma*-like lung lesions were observed in two piglets from the NC group at necropsy (D28), even though these piglets tested *M. hyopneumoniae*-negative by dPCR (D14 and D28) and did not seroconvert (D0 and D28). All piglets in the different infected groups (S1, S2, and S3) developed *Mycoplasma*-like lung lesions by D28. The mean MLCL and MLL scores were significantly higher in the *M. hyopneumoniae* group in comparison to the NC group *($p < 0.05$)*, while the percentage of air in the lung tissue was not statistically different between both groups (Table 7). The average values for MLCL and MLL scores, the percentage of air, and other parameters of the groups NC, S1, S2, and S3 are shown in Additional file 9.

Detection of M. hyopneumoniae DNA in BALF and specific antibodies in serum

All control animals tested negative for *M. hyopneumoniae* DNA (D14 and D28), while all infected animals were positive (Additional file 9) and an increase in the DNA load was detected from D14 to D28 ($p < 0.05$). All animals were seronegative on D0. The control animals remained negative until D28, while the *M. hyopneumoniae*-infected animals seroconverted by D28, except for one animal from the S2 group and one from the S3 group. Additionally, a statistically significant difference *($p < 0.05$)* between NC and the *M. hyopneumoniae* group was reported for DNA load (D14 and D28) and antibody value (D28), as shown in Table 7.

Associations between the evaluated parameters

Significant correlations were reported among most parameters assessed in this study, as shown in Table 8. A very strong ($r > 0.80$) negative correlation was observed between MLL and percentage of air, while strong positive correlations ($r = 0.60$ to 0.80) were present between MLCL, RDS post-inoculation, *M. hyopneumoniae*-DNA, and anti-*M. hyopneumoniae* antibodies. Positive and moderate correlations ($r = 0.30$ to 0.59) were found between MLCL, MLL, *M. hyopneumoniae*-DNA, RDS post-inoculation, and anti-*M. hyopneumoniae* antibodies. Lastly, no significant correlations were observed for the IMT post-inoculation data and the other clinical parameters, besides a moderate correlation between IMT and RT ($r = 0.49$; $p>0.05$).

Table 8. Spearman's correlation coefficient matrix of the main clinical parameters. RT: rectal temperature; MLCL: macroscopic lung consolidated lesion; MLL: microscopic lung lesion; IMT pi: intramuscular temperature post-inoculation (D0 to D28); RDS pi: respiratory disease score post-inoculation (D0 to D28); D: day.

Spearman's rho	*RT (D-7 to D-3)*	*MLCL (D28)*	*MLL (D28)*	*IMT pi (D0 to D28)*	*RDS pi (D0 to D28)*	*% of air (D28)*	*M. hyopneumoniae-DNA (D14)*	*M. hyopneumoniae-DNA (D28)*
IMT (D-7 to D-3)	0.490 *	N.A	N.A	N.A	N.A	N.A	N.A	N.A
MLL (D28)	N.A	0.572 *	-	-	-	-	-	-
IMT pi (D0 to D28)	N.A	0.371	0.239	-	-	-	-	-
RDS pi (D0 to D28)	N.A	0.646 *	0.420 *	0.045	-	-	-	-
% of air (D28)	N.A	-0.344	-0.841 *	-0.265	-0.097	-	-	-
M. hyopneumoniae-DNA (D14)	N.A	0.586 *	0.368	0.035	0.667 *	-0.106	-	-
M. hyopneumoniae-DNA (D28)	N.A	0.631 *	0.469 *	0.061	0.701 *	-0.154	0.758 *	-
anti-M. hyopneumoniae antibodies (D28)	N.A	0.734 *	0.533 *	0.134	0.725 *	-0.203	0.679 *	0.743 *

* Significant correlation, $p < 0.05$

The strength of the correlations (rho) is indicated by the intensity of the blue color. N.A: not applicable.

Discussion

The present study showed that *M. hyopneumoniae* infection reduced movement and as expected, increased MLCL, MLL, RDS, DNA load, and anti-*M. hyopneumoniae* antibodies levels. Percentage of air, ADWG, and IMT were not affected. It was also shown that intramuscular thermochips can be an alternative tool to assess body temperature under experimental conditions. Furthermore, contemporary *M. hyopneumoniae* isolates (11.1A and 13.1B) showed similar results on the main clinical parameters, such as MLCL,

MLL, RDS, *M. hyopneumoniae*-DNA load, and anti-*M. hyopneumoniae* antibodies, to those observed with the reference *M. hyopneumoniae* strain F7.2C (S1).

Most infectious diseases can decrease the activity and movement behavior of animals (Hart, 1988; Reiner et al., 2009; Rostagno et al., 2011), making this information useful for welfare monitoring and early disease detection. In this study, changes in the movement were seen from D2 onwards, preceding the changes in the RDS after D8. In addition, we observed a lower percentage of movement in the *M. hyopneumoniae*-infected group and a higher chance of movement in the control group, confirming the hypothesis that *M. hyopneumoniae* infection negatively influences the movement of pigs. Similar results were reported for PRRSv infection in pigs. The piglets in the PRRS-positive group moved less, and also spent less time feeding when compared to the control group (Escobar et al., 2007). However, in that previous study, both PRRS-negative and PRRS-positive animals were also infected with *M. hyopneumoniae*, indicating that PRRSv infection further decreased movement in *M. hyopneumoniae*-infected animals. It is believed that, in the present study, the infectious agent (*M. hyopneumoniae*) was the main pathogen affecting the pigs' movement as the animals originated from the same specific pathogen free-herd and strict biosecurity measures were applied between the rooms, throughout the entire study period. Nonetheless, investigating the movement of *M. hyopneumoniae*-positive and negative herds or batches would be essential for a comprehensive understanding of the movement behavior of pigs under field conditions.

The activity and movement patterns of pigs can be influenced by various factors, such as the space allocated per animal, housing conditions (including floor type, temperature, and access to toys), as well as age and weight (Docking et al., 2008; Luo et al., 2020; Petersen et al., 1995). Studies have indicated a decline in walking and standing behavior with age, primarily attributed to the limited space available per pig, a factor directly impacted by the pig's growth (Docking et al., 2008; Petersen et al., 1995). In our study, the piglets were kept in separate, but very similar rooms (same size, floor, heating, same number and location of toys and feeders), and with the same feed and water management. Interestingly, in our study, the movement of piglets appeared not to be

affected by age or space allocation per pig, possibly due to the relatively brief duration of our observation period (seven weeks) and the available floor area per pig.

In our study, piglet movement behavior was negatively affected by *M. hyopneumoniae* infection, suggesting that this parameter could be used for respiratory disease detection in a more chronic setting alongside well-established parameters such as MLCL, RDS, DNA load, and *M. hyopneumoniae*-specific antibodies. The main advantage of such a system is that it can then be done in an objective and quantitative way. Besides, the continuous assessment of movement on farms could also be highly valuable to veterinarians, serving as a potential tool for monitoring and managing pig herd health, enabling early disease outbreak detection. On top of that, if algorithms had the capacity to discriminate between animals infected with specific pathogens, such as *M. hyopneumoniae* and PRRSv, by analyzing changes in movement, it might transform the landscape of personalized medicine within agricultural contexts, leading to groundbreaking progress. Even though our results encourage further research into this topic, changes in movement alone should be interpreted with caution for diagnostic purposes, especially because it can be influenced by many factors, and not only by infections with pathogens.

Environmental conditions, such as ammonia, carbon dioxide, dust particles, temperature, humidity, and ventilation rate are known risk factors for the development and aggravation of respiratory diseases in pigs (Pieters and Maes, 2019; Yaeger and Alstine, 2019), and may also influence their movement activity (Kim et al., 2008). In this study, similar environmental conditions were observed between the groups, except for the dust particles (10 µg/m^3) and sound levels at 602 Hz. Higher values were observed for the NC group as compared to the infected groups. The greater values of dust particles observed in the NC group may be related to the movement activity of the pigs, although other factors such as an increased feed spill could also have played a role. Regarding the sound level data, numerical differences were observed within the 602 Hz frequency, where animals in the NC group produced more intense sounds throughout the study when compared to the infected groups. Although lower sound frequencies (400 - 600 Hz) have been associated with coughing caused by infectious agents, such as *Pasteurella multocida* (Ferrari et al.,

2008), the numerical difference observed in the 602 Hz frequency between NC and *M. hyopneumoniae*-infected groups should be interpreted with caution, especially because the difference was already reported before the inoculation of the animals, indicating that other factors such as housing conditions or malfunctioning of the sound sensors could have played a role.

The main clinical sign of *M. hyopneumoniae* infection in pigs is the non-productive cough. However, body temperature (Almeida et al., 2020c) and performance parameters, such as ADWG, can also be affected (Ferraz et al., 2020). The onset of the coughing starts between one and two weeks after infection and increases up to five weeks (Pieters and Maes, 2019). Not surprisingly, RDS data were significantly higher in the infected animals from 10 days post-inoculation (dpi) to 28 dpi as compared to the control animals, corroborating previous studies (Almeida et al., 2020b; Michiels et al., 2017a; Michiels et al., 2018; Vicca et al., 2003b). These increased RDS values can be directly associated with the development of the disease and its inflammatory process (Pieters and Maes, 2019). This is also supported by the strong correlations between RDS post-inoculation and MLCL, *M. hyopneumoniae*-DNA, and anti-*M. hyopneumoniae* antibodies. Similar results were reported when different *M. hyopneumoniae* strains and challenge models were used (Almeida et al., 2020c; Vicca et al., 2003a). Concerning ADWG, no differences were observed between the groups in the different periods in our study, which is likely due to the rather short duration of the study and the limited number of animals used (Vicca et al., 2003a).

Assessment of the RT in pigs is often used in experimental trials but is not very convenient when repetitive measurements must be taken. In addition, measuring RT may not reflect the temperature at rest, as it might increase due to the movement and possible stress of handling. This might also result in a high variation of temperature data between pigs. Thus, alternative methods, such as thermal transponders and portable readers, are of increasing interest (Jara et al., 2016; Lohse et al., 2010; Suli et al., 2017). When microchips were placed at distinct locations of the body, such as subcutaneous, intramuscular, or intraperitoneal implantation (Allerson and Morrison, 2011; Burdick Sanchez et al., 2023; Jara et al., 2016; Lohse et al., 2010; Suli et al., 2017), different

results were reported. In our study with intramuscularly implanted microchips, a good correlation between IMT and RT was observed, and median IMT values were 0.25 °C higher than the corresponding median RT values. Previous studies also reported a correlation between RT and IMT, although the average IMT values were slightly lower than the RT values (Allerson and Morrison, 2011; Suli et al., 2017). This could have been attributed to an increase in temperature just before RT measurement (possibly due to handling and stress), and also to the location of the microchip, as microchips implanted subcutaneously lead to lower values in comparison to RT data (Lohse et al., 2010). The IMT did not differ significantly between *M. hyopneumoniae*-infected and NC groups, which might be due to the low sample size and measurement frequency. Nonetheless, considering the ease of use and good correlation with RT, thermal microchips seem to be a convenient and accurate alternative to assess body temperature of pigs under experimental conditions.

Although not pathognomonic, cranioventral pulmonary consolidations in the lung are suggestive of *M. hyopneumoniae* infection in pigs (Pieters and Maes, 2019). Likewise, broncho-interstitial pneumonia with hyperplasia of bronchus-associated lymphoid tissue (BALT) lesions is characteristic of *M. hyopneumoniae* infection. In our study, macroscopic and microscopic lesions differed significantly between infected and control animals. The MLCL were moderate to strongly associated with MLL, *M. hyopneumoniae*-DNA load, and anti-*M. hyopneumoniae* antibodies. A very strong and negative correlation between MLL and the percentage of air was reported in our study, which is logic as the presence of lymphocytes, macrophages, and neutrophils increases with stronger inflammatory responses, and consequently decreases the percentage of air in the alveoli (Beuckelaere et al., 2022; Meyns et al., 2007; Pieters and Maes, 2019). Despite the strong correlation, no significant differences were observed for percentage of air between the NC and *M. hyopneumoniae*-infected groups, which could be due to the great variability of the data. Thus, as MLL holds more intrinsic and direct information concerning the degree of inflammation when compared to the percentage of air, we prefer the assessment of MLL over assessing the percentage of air in the lung tissue.

Lastly, regarding the production of *M. hyopneumoniae*-specific antibodies upon *M. hyopneumoniae* infection, strong correlations between anti-*M. hyopneumoniae* antibody levels and the key clinical parameters (MLCL, MLL, RDS, and DNA load) were observed. Most of the infected animals (16/18) seroconverted by D28. Previous studies reported higher numbers of piglets seroconverting by 28 dpi when using highly virulent strains compared to low virulent strains (Meyns et al., 2007; Vicca et al., 2003a). In addition, based on the similar results described for the infected groups, we believe that these contemporary *M. hyopneumoniae* isolates (strain 11.1A and 13.1B) might have a virulence similar to the F7.2C strain. However, since the piglets were also challenged with a low virulent strain (F1.12A), further research is needed to assess the virulence of the new isolates in the absence of a subsequent challenge.

While our findings are important and can be useful to monitor *M. hyopneumoniae* infection in pigs, some limitations should be mentioned. First, the study was done under experimental conditions with a limited number of animals and a rather short duration. As such, the results do not reflect the field situation, where animals are kept for a longer period, are subjected to different housing and management conditions, and have different sanitary challenges. These conditions could also influence the movement of pigs, making it more difficult to associate the change in movement with a specific pathogenic infection. Furthermore, the use of thermal chips to assess body temperature in the field is currently seldom used, possibly due to the risk of becoming a hazard in the meat, as locating it during the slaughter process would not be feasible. Lastly, although no added value regarding weight gain was observed in our study, ADWG remains of great relevance, particularly for long-term studies, field experiments, and the swine industry in general.

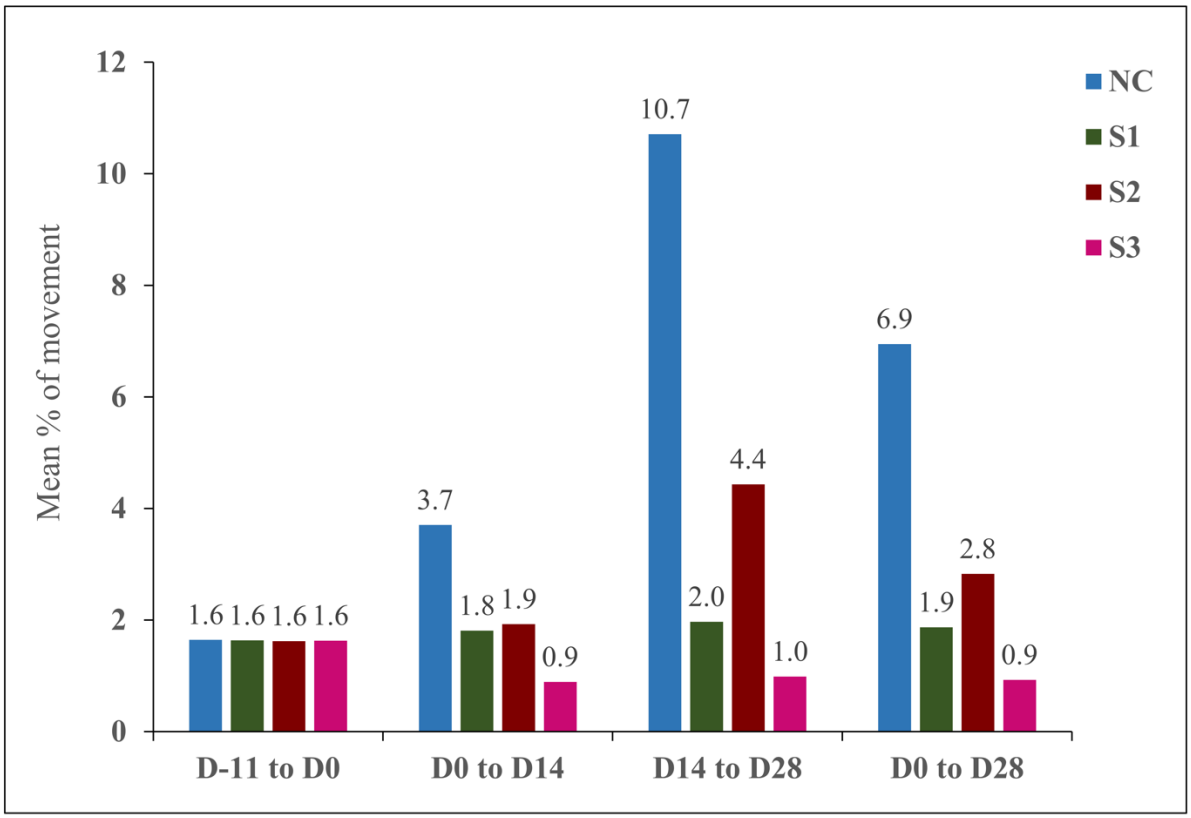
Therefore, considering the aforementioned results, we suggest that a specific set of parameters, comprising movement, macroscopic and microscopic lung lesion scores, coughing (RDS), bacterial load, and antibody levels, can be used to efficiently monitor *M. hyopneumoniae* infection in pigs under experimental conditions; and that percentage of air in the lung and ADWG have no added value, especially for rather short studies. Thus, the present study provides critical insights in which parameters could be evaluated upon *M. hyopneumoniae* infection to reach a maximum efficiency of used resources under

experimental conditions. Further research is nevertheless needed to investigate the outcome of these parameters in different study conditions.

Conclusions

The present study showed that the movement of pigs was negatively affected by experimental infection with *M. hyopneumoniae*. Body temperature data assessed using microchips was on average 0.25°C higher than but correlated well with data from rectal temperature measurements. Moderate to strong correlations were observed between all parameters assessed in this study, except for IMT and movement post-inoculation. Based on our findings, we suggest the use of movement data for early disease detection, followed by the comprehensive evaluation of a specific set of parameters—specifically macroscopic and microscopic lung lesion scores, coughing (RDS), bacterial load, and antibody levels—to assess *M. hyopneumoniae* infection in pigs.

Additional Files

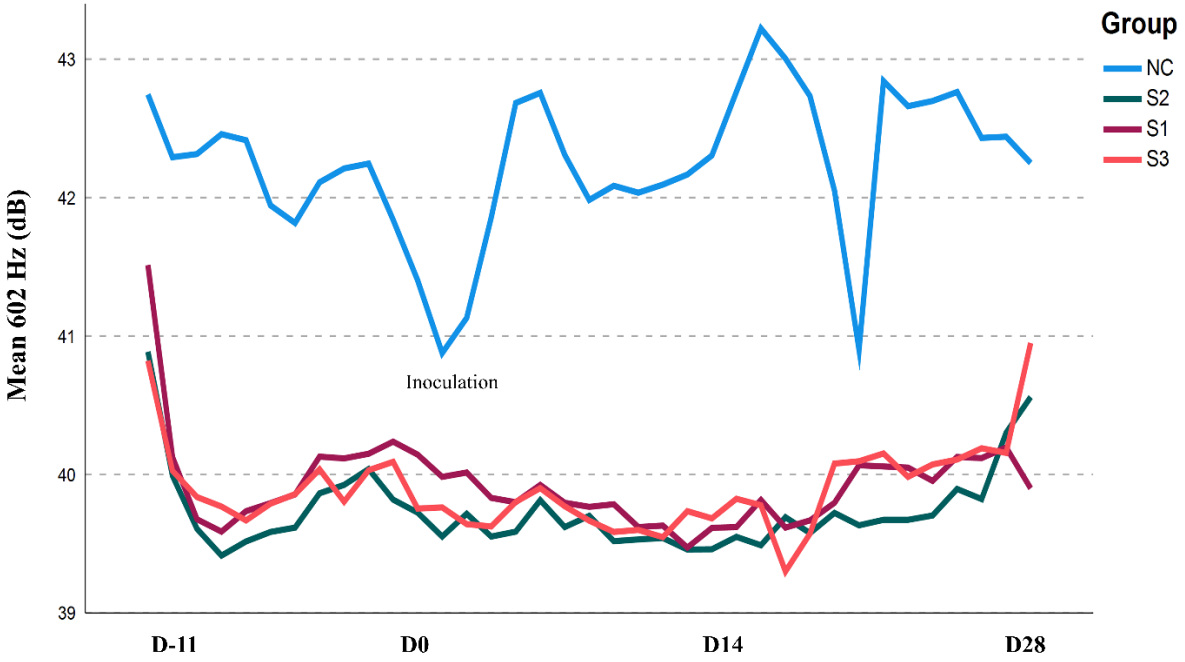


Additional file 4. **Figure.** Mean percentage of movement (y axis) in the experimental groups according to the study period (x axis). NC: Negative control group; S1: group infected with the *M. hyopneumoniae* strain F7.2C; S2: group infected with *M. hyopneumoniae* strain 11.1A; S3: group infected with *M. hyopneumoniae* strain 13.1B; D: day.

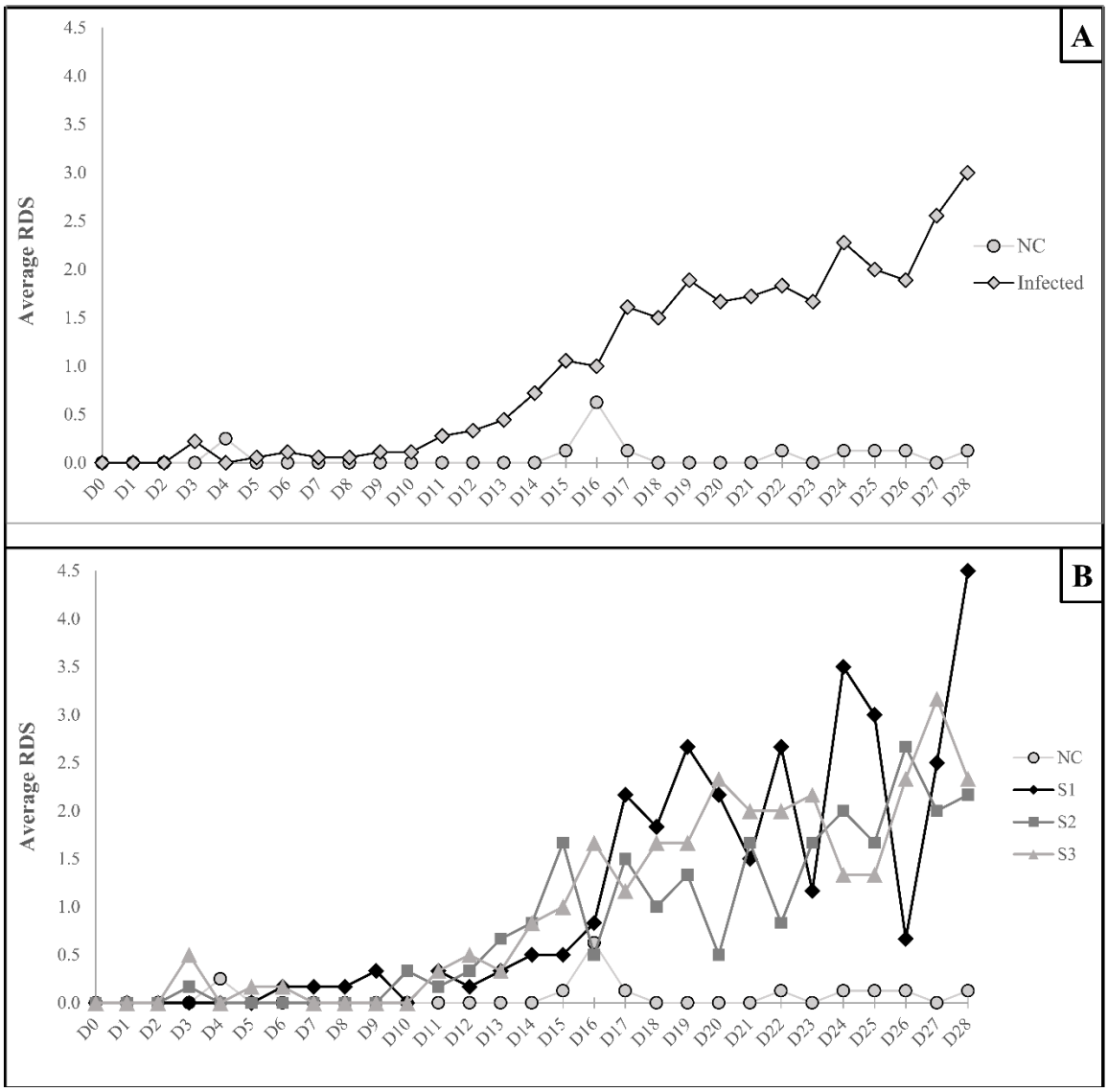
Additional file 5. Table. Detailed values on the main environmental parameters measured by the Healthy Climate Monitor throughout the study (D-12 to D28). NC: Negative control group; S1: group infected with the F7.2C *M. hyopneumoniae* strain; S2: group infected with the recently isolated 11.1A *M. hyopneumoniae* strain; S3: group infected with the recently isolated 13.1B *M. hyopneumoniae* strain; D: day.

Parameter	Group	Median	Min.	Max
Sound 602 Hz (dB)	NC	42.20	39.00	51.30
	S1	39.60	39.00	48.80
	S2	39.30	39.00	45.70
	S3	39.50	39.00	49.70
CO_2 (ppm)	NC	571.60	347.00	1030.90
	S1	618.80	522.20	1192.60
	S2	631.55	512.90	867.60
	S3	717.40	617.00	950.60
NH_3 (ppm)	NC	0.00	0.00	3.30
	S1	0.00	0.00	2.20
	S2	0.00	0.00	4.50
	S3	0.00	0.00	3.30
Ambient temperature (°C)	NC	25.00	21.20	26.60
	S1	24.00	19.80	25.60
	S2	24.40	20.60	28.30
	S3	24.00	21.10	27.50
Relative humidity (%)	NC	34.90	24.30	52.40
	S1	34.60	24.60	53.20
	S2	38.80	28.00	57.20
	S3	29.40	14.10	46.90
Air pressure (hPa)	NC	1018.70	983.90	1031.40
	S1	1019.00	983.90	1031.60
	S2	1019.60	984.10	1031.70
	S3	1019.10	984.20	1031.80
PM 10 (µg/m3)	NC	44.60	37.50	203.20
	S1	2.00	1.00	42.00
	S2	1.00	1.00	11.50
	S3	1.00	1.00	13.80
PM 1 (µg/m3)	NC	44.40	37.50	202.90
	S1	1.10	0.00	41.00
	S2	0.85	0.00	3.00
	S3	1.00	0.00	7.60

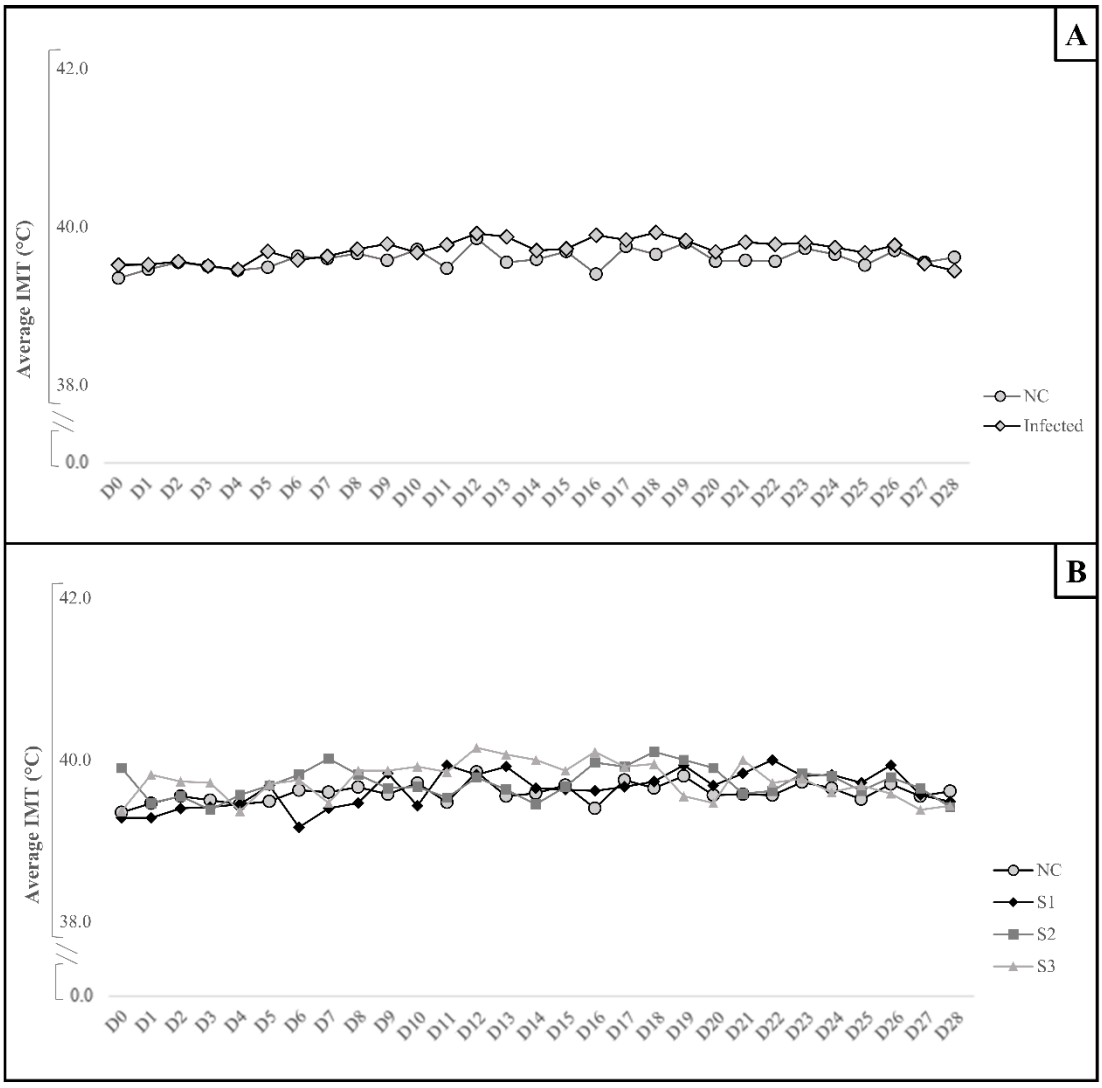
dB= decibels; ppm= parts per million; hPa= hectopascal; PM: particle matter



Additional file 6. Figure. Mean values of the 602 Hz frequency by group, where the daily average was calculated based on several measurements in the 24-hour period. Overall, higher values were observed in the negative control group. NC: Negative control group; S1: group infected with *M. hyopneumoniae* strain F7.2C; S2: group infected with *M. hyopneumoniae* strain 11.1A; S3: group infected with *M. hyopneumoniae* strain 13.1B. D: day.



Additional file 7. Figure. Average RDS data by treatment (A) and by the individual groups (B). NC: Negative control group; S1: group infected with the F7.2C *M. hyopneumoniae* strain; S2: group infected with the recently isolated 11.1A *M. hyopneumoniae* strain; S3: group infected with the recently isolated 13.1B *M. hyopneumoniae* strain; D: day.



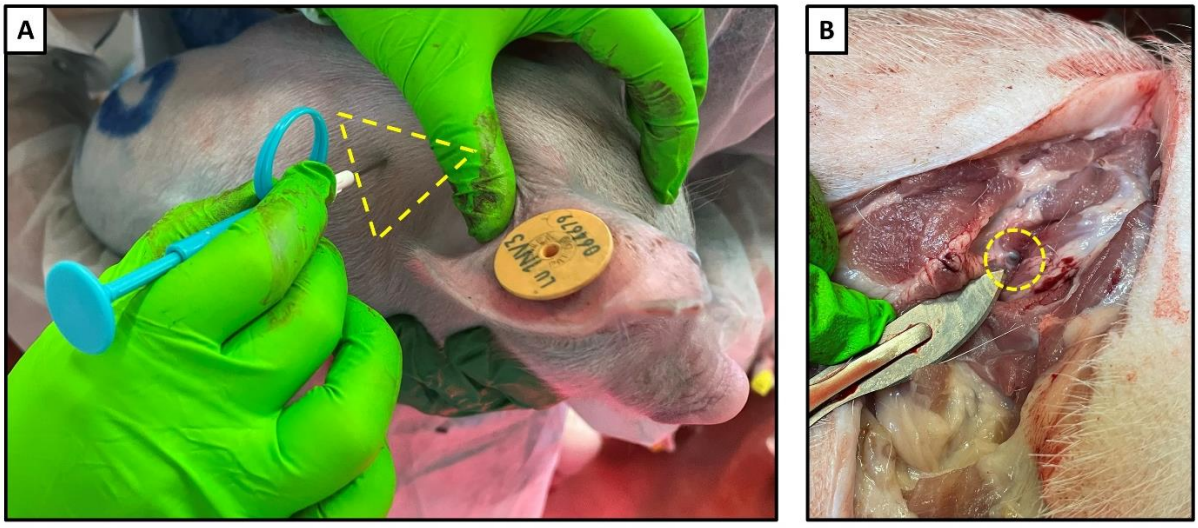
Additional file 8. Figure. Average IMT data by treatment (A) and by the individual groups (B). NC: Negative control group; S1: group infected with the F7.2C *M. hyopneumoniae* strain; S2: group infected with the recently isolated 11.1A *M. hyopneumoniae* strain; S3: group infected with the recently isolated 13.1B *M. hyopneumoniae* strain; IMT: intramuscular temperature, D: day.

Additional File 9. Table. Mean and standard deviation (sd) of the main clinical parameters evaluated by group. RDS: respiratory disease score; BT: body temperature; ADWG: average daily weight gain; MLCL: macroscopic lung consolidated lesion; MLL: microscopic lung lesion.

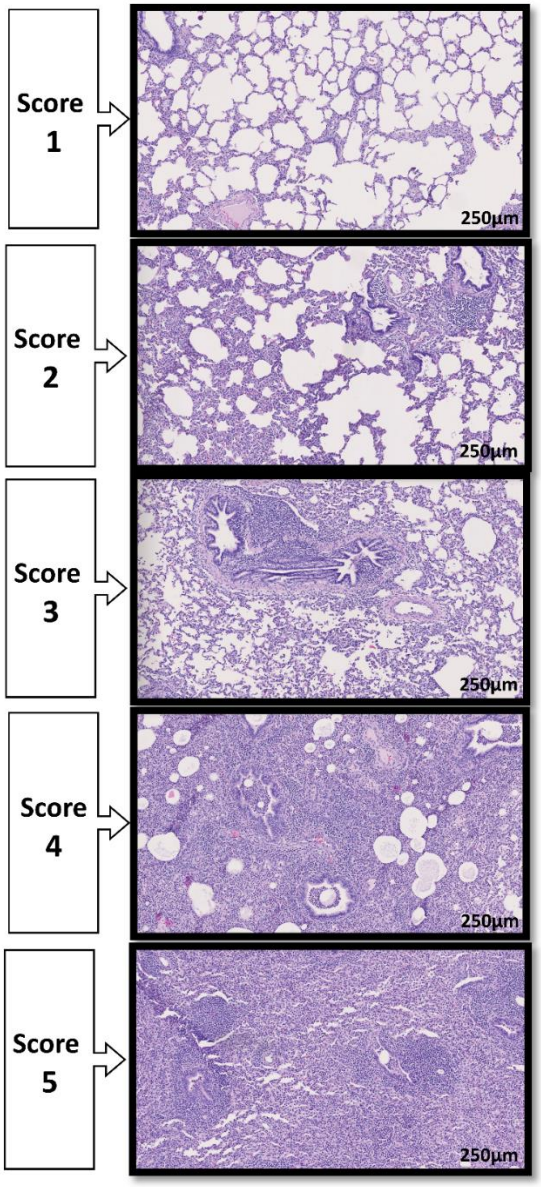
Parameter	Study day	NC		S1		S2		S3	
		mean	sd	mean	sd	mean	sd	mean	sd
RDS	D-11 to D0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	D-11 to D28	0.0	0.1	0.8	0.4	0.6	0.4	0.7	0.3
	D0 to D28	0.1	0.2	1.1	0.9	0.8	0.9	1.0	0.7
	D14 to D28	0.1	0.1	2.0	0.5	1.5	0.5	1.8	0.4
BT in °C	D0 to D5	39.5	0.2	39.4	0.2	39.6	0.2	39.6	0.3
	D0 to D10	39.6	0.2	39.4	0.2	39.7	0.2	39.7	0.3
	D0 to D28	39.6	0.2	39.6	0.1	39.7	0.2	39.7	0.3
ADWG in g/pig/day	D-12 to D0	114	48	142	58	119	28	80	57
	D-12 to D28	328	90	351	97	414	42	322	110
	D0 to D28	419	124	440	132	540	57	426	141
M. hyo-antibody values	D0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	D28	0.0	0.0	0.9	0.2	0.6	0.3	0.6	0.3
M. hyo-DNA load (log10)	D14	0.0	0.0	4.0	0.8	3.5	0.2	4.1	0.4
	D28	0.0	0.0	4.7	0.3	4.3	0.3	4.3	0.4
MLCL score	D28	0.6	1.3	3.9	1.8	4.4	2.5	4.7	2.9
MLL score	D28	2.2	0.2	2.4	0.2	2.7	0.4	2.8	0.4
Percentage of air in lung tissue	D28	37.3	4.5	37.4	5.9	33.4	5.7	32.9	6.8

NC: Negative control group; S1: group infected with the F7.2C *M. hyopneumoniae* strain; S2: group infected with the recently isolated 11.1A *M. hyopneumoniae* strain; S3: group infected with the recently isolated 13.1B *M. hyopneumoniae* strain; D: day.

Additional File 10. **Picture.** Image obtained from the Healthy Climate Monitor showing a complete view of the negative control room.



Additional File 11. **Picture.** Thermal microchip implantation in pigs; A) microchip being implanted on the right side of the neck behind the base of the ear (dashed triangle); B) post-mortem location of the microchip is indicated by the dashed circle.



Additional File 12. **Picture.** Microscopic lung lesion scoring adapted from Morris et al. (1995a) and Vicca et al. (2003a). Score 1: limited infiltration of macrophages and lymphocytes around bronchioles, with airways and alveolar spaces free of cellular exudates; Score 2 = light to moderate infiltrates with mild diffuse cellular exudates into airways; Scores 3, 4, and 5 (mild, moderate and severe, respectively) lesions characteristic of broncho-interstitial pneumonia, centered around bronchioles but extending to the interstitium, with lymph follicular infiltration and mixed inflammatory cell exudates. The pictures as shown as 10X magnification.

Chapter 5 | General discussion

Over the past years, new technologies like high-throughput DNA sequencing and smart sensors have been introduced and explored in animal research. For instance, the respiratory microbiome of pigs has been further investigated thanks to the increasing accessibility of sequencing technologies. Yet, researchers illustrated that the microbiome can be modulated by several factors, including pathogens, the environment and antimicrobial usage, among others (Blanco-Fuertes et al., 2023; Pirolo et al., 2021; Zeineldin et al., 2018). Furthermore, with the introduction of artificial intelligence, the use of sensors and real time-monitoring is becoming more feasible in swine production, as the interpretation of the big data files can be (partly) taken over by AI-driven software.

To date, little is known about the influence of *M. hyopneumoniae* infection on the respiratory microbiome and on the movement of pigs, which can be measured using sequenced-based technologies and image-based sensors, respectively. Furthermore, in the near future, these technologies might be important for management of animal health and control of diseases. Therefore, the general objective of this thesis was to investigate the lung microbiome of pigs infected with and free of *M. hyopneumoniae*, as well as other factors (like movement) as potential supportive parameters in the diagnosis of *M. hyopneumoniae* infections.

In **Chapter 3**, we investigated the influence of natural *M. hyopneumoniae* infection on the microbiome of pigs. In addition, we assessed the association of *M. hyopneumoniae, M. flocculare*, and *M. hyorhinis* with lung lesions. In **Chapter 4**, we assessed certain parameters, such as animal movement and body temperature measured using microchips in *M. hyopneumoniae*-infected and non-infected pigs and whether the currently used diagnostic parameters for *M. hyopneumoniae* can be rationalized, especially in experimental research.

5.1. Influence of *M. hyopneumoniae* infection on the respiratory microbiome

It has been hypothesized that *M. hyopneumoniae* infection alters the lung microbiota of pigs, favoring the multiplication of other pathogens (Ciprián et al., 1988; Pirolo et al., 2021; Siqueira et al., 2017), and with increasing accessibility of sequencing methods, this idea was further explored. The study in Chapter 3 showed that *M. hyopneumoniae*:

1. correlated with the severity of macroscopic lung lesions.
2. influenced the microbial diversity, with infected animals showing a lower bacterial diversity compared to non-infected ones.
3. might worsen MLCL when other pathogens are involved, particularly with *P. multocida*.

The study also showed that BALF samples were more valuable than nasal turbinates swabs for assessing associations between *M. hyopneumoniae* infection and the respiratory microbiota.

Altogether, the presence of *M. hyopneumoniae* in BALF was associated with changes in the bacterial community of the LRT. An *M. hyopneumoniae* infection may lead to dysbiosis and proliferation of other pathogens in the lung and possibly in the URT, and may lead to increased severity of lung lesions, as previously reported (Pirolo et al., 2021; Rodriguez et al., 2020; Siqueira et al., 2017). However, as the animals were investigated at slaughter, at one timepoint, it was not possible to conclude whether the microbiome could have influenced the development of *M. hyopneumoniae* infection. More detailed longitudinal studies are warranted to explore this. Additionally, when taking a closer look at the findings related to the nasal turbinate samples, low relative frequencies of *M. hyopneumoniae* and higher values for alpha diversity were reported, suggesting a greater diversity of genera when compared to BALF samples. Even though the URT microbiome influences the LRT microbiome in healthy individuals, and likely also influences the LRT microbiome in case of disease (Li et al., 2024; Whiteside et al., 2021), it is not yet known how this interaction occurs in pigs or what its relative importance is in the pathogenesis of respiratory disease.

5.1.1. Applicability of microbiome data in research and field settings

Understanding the influence of the microbiome on respiratory health in pigs, especially in relation to *M. hyopneumoniae* infection, is important to be able to extrapolate the results and their applicability to experimental and field conditions.

It has been suggested that the respiratory microbiome of pigs changes significantly up to 3 weeks after weaning (Slifierz et al., 2015). In addition, these authors also mention

that minimizing the exposure to antibiotics and the contact with potential pathogens during early-life might allow the development of a resilient microbiome (Slifierz et al., 2015). Researchers also suggested that the abundance of lactobacilli may influence host immunity and disease susceptibility due to the immunomodulatory activity of some bacteria in the gut microbiome of piglets (Schokker et al., 2014). In addition, recent studies have suggested a communication between the lung and gut microbiome, known as the gut-lung axis (Enaud et al., 2020). In humans, Hoen et al.(Hoen et al., 2015) suggested that changes in the gut microbiota, such as a decrease of *Parabacteroides*, are predictive of airway colonization with *P. aeruginosa.* Thus, strategies such as early-life exposure to beneficial microbes, probiotic supplementation, and optimizing environmental conditions might help shape a resilient and diverse respiratory microbiome.

Moreover, if certain components of the microbiome are found to negatively influence respiratory health, they could serve as diagnostic targets, helping veterinarians to identify early indicators of respiratory diseases, and to implement targeted interventions as soon as possible. Understanding parts of the microbiome that positively influence respiratory health opens avenues for microbiome-based interventions to prevent or mitigate severe respiratory diseases such as PRDC. For instance, promoting the growth of beneficial bacteria through prebiotics might help modulate the microbiome towards a healthier, more diverse composition. In addition, minimizing stressors like transportation, overcrowding, poor ventilation, and exposure to a variety of pathogens in the early life, might help to stabilize the respiratory microbiota, making the animals less susceptible to respiratory infections and to treatment with antibiotics.

The microbiome can be explored to identify possible next generation of probiotics that could be used to rebuild or reinforce tolerance and prevent infections resulting from highly antibiotic-resistant bacteria (Li et al., 2024). In humans, the role of oral probiotics in preventing URT infections was evaluated, with evidence of their beneficial effects (Al-Romaih et al., 2023; Popova et al., 2012). Recently a study by Blanco-Fuertes et al. (Blanco-Fuertes et al., 2023) showed that when ceftiofur was given to sows before farrowing, it had a longer-lasting effect on the composition of the nasal microbiota in piglets than when it was delivered directly to the young animals. In addition, the

researchers showed, for the first time, that nasal inoculation (spray) of natural colonizers of the URT modified the piglets' nasal microbiota to a more stable scenario and better clinical status after weaning (Blanco-Fuertes et al., 2023). Lastly, as this is almost an unexplored field in animals, and especially in pigs, exploring the complexity of the respiratory microbiome and the influence of antimicrobials and selected colonization might be essential for developing alternative strategies for disease prevention, diagnosis, and treatment in pigs.

5.1.2. General considerations and further research

While our results provide valuable insights into the respiratory microbiome in *M. hyopneumoniae*-infected pigs, the following should be acknowledged and should be taken into account when performing similar studies in the future:

1. The study lacked sufficient evaluation of the negative controls, making it challenging to attribute observed changes in the bacterial community solely to *M. hyopneumoniae* infection. Furthermore, the negative controls (collected during sampling and DNA extraction) tested negative for *Mycoplasma* by real time PCR. However, the possibility of background contamination in the microbiome analysis cannot be ruled out, as these samples were not sequenced and because of the low biomass of the specimens, especially BALF. Last, standardization of sample processing procedures and use of reagents from the same batch might have helped in maintaining consistency and reliability across the samples.
2. The study's scope may have been limited by the inclusion of only three farms. Thus, variation between farms, such as management practices, environmental conditions, and pig genetics, could have impacted the results.
3. Sample collection at the slaughterhouse introduced the risk of cross-contamination from environmental sources, such as the scalding water and the machinery, as observed in the NT sample from the negative farm (H1). As already suggested by Marois et al. (2008), DNA from pathogens can be detected in the scalding tank even before the onset of slaughter, and because of the direct contact of the water with the URT (mainly), it might serve as source of cross contamination.

4. Using only one pooled sample per farm may not have captured the variability within the respiratory microbiota. Thus, individual variability among pigs within the same farm could not be evaluated in our study.

Addressing and acknowledging these experimental limitations are essential steps in ensuring the validity and reliability of the findings. Further research with larger sample size and more robust controls will enhance our understanding of the respiratory microbiota dynamics in pigs.

Also, further research could investigate whether vaccination against *M. hyopneumoniae* or other respiratory pathogens modulates the respiratory microbiome of pigs (or vice versa) and influences the composition, diversity, and stability of the respiratory microbiota. In that sense, a study using a mouse model (Oh et al., 2014) showed that the microbiota was essential for inducing short-lived and long-lived plasma cell responses after vaccination against influenza, with impaired memory B cell recall, suggesting a necessity for diverse microbial communities rather than specific bacterial species. In addition, it has been demonstrated that the microbiota is a constant source of natural adjuvants, which are capable of activating pathways that control innate and adaptive immunity (Ciabattini et al., 2019; Pabst and Hornef, 2014). Such studies provide some insights into the mechanisms underlying vaccine-mediated protection against *M. hyopneumoniae*.

In addition, exploring whether different strains of *M. hyopneumoniae* result in distinct changes in the respiratory microbiome might also be important to understand strain-specific host-microbiome interactions. Finally, investigating the potential of commensal bacteria, such as *M. flocculare* or *Lactobacillus*, as probiotics to modulate the respiratory microbiome, potentially enhancing respiratory health, and as such reducing antimicrobial use in pigs could be a promising area of research (Zeineldin et al., 2018).

5.2. *M. hyopneumoniae* infection: standard parameters and new tools

Although *M. hyopneumoniae* infection is considered to impact behavior (Escobar et al., 2007), especially movement, no studies have been conducted to further investigate this hypothesis. Thus, in Chapter 4, we investigated the movement and body temperature

of pigs, along with other established parameters upon experimental *M. hyopneumoniae* infection. The following results were obtained:

1. *M. hyopneumoniae* infection significantly alters the movement of piglets, suggesting that movement might be a useful parameter for monitoring and detecting early signs of disease in pigs, even when this disease is developing more slowly than other diseases e.g. acute pleuropneumonia outbreaks.
2. Intramuscular thermochips prove to be an alternative and accurate tool for assessing body temperature in pigs under experimental conditions, showing a good correlation with rectal temperature. This is especially useful for experimental studies on chronic conditions, as for *M. hyopneumoniae* infection, where subtle changes in body temperature are expected, especially after challenge or vaccination.
3. Conventional parameters used in research such as macroscopic and microscopic lung lesion scores, respiratory disease scores (RDS), *M. hyopneumoniae* DNA load, and anti-*M. hyopneumoniae* antibody levels are significantly affected by *M. hyopneumoniae* infection, providing valuable insights into the progression and severity of the disease, especially in a research setting.
4. Parameters such as the percentage of air in histological examination of lung tissue and body weight gain do not seem to have added value for (short) experimental trials with *M. hyopneumoniae,* suggesting that these parameters could be left out or be replaced by other parameters in research settings.

Overall, the study highlights the potential use of movement as a monitoring tool for *M. hyopneumoniae* infections, and the use of intramuscular thermochips for temperature assessment as an alternative to rectal temperature.

5.2.1. Applicability of conventional parameters under field and research settings

Monitoring pig movement, in which changes in patterns, such as reduced activity, might serve as a valuable indicator of pig health. As *M. hyopneumoniae* is a primary pathogen and is known to facilitate infection by secondary pathogens, likely worsening the disease, being able to identify pathological conditions early on might help with

immediate treatment. Additionally, it can provide objective and quantitative measures, enhancing the accuracy and reliability of the data (Xu et al., 2023; Zhou et al., 2023). Yet, processing of the data happens almost instantaneously, allowing the farmers to have the results immediately. Monitoring movement might complement traditional clinical parameters like coughing frequency, lung lesion scores, and antibody levels, offering a more comprehensive assessment of the disease course. Thus far, most studies evaluating the movement and behavior of pigs were conducted under experimental conditions (Escobar et al., 2007; Matthews et al., 2017; Pandey et al., 2021; Rostagno et al., 2011). A robust study under field conditions conducted by Nasirahmadi et al. (2019) showed that their model/algorithm has flexibility and good robustness towards different lighting conditions, ages and skin color, being an important step towards developing real-time, continuous computer-based monitoring for farms. Recently, commercial tools are becoming available on the market, such as the Healthy Climate Monitor (Solutions.) and Pig Move (Asserva), which might be used to monitor pig movement at the farm level.

Even though variable results are observed when microchips are implanted subcutaneously (Lohse et al., 2010), the use of microchips implanted intramuscularly seems to offer accurate temperature data, correlating very well with rectal temperature measurements (Allerson and Morrison, 2011). But, regardless of where the microchips are implanted, they offer the possibility to record repeated measurements without the need to handle the animals. This is convenient and saves labor and time for the researchers. Also, it is animal friendly as the animals do not need to be restrained. In experimental settings, where precise temperature monitoring is often important, thermal microchips offer a reliable method for assessing physiological responses to infection or stressors. Suli et al. (2017) observed a decrease in animal's motion when the body temperature increases, suggesting that temperature could be integrated with other clinical parameters. Even though microchips can be easily used in research settings, their use in pigs on commercial farms might be difficult. It is possible that microchips migrate in the body (Lohse et al., 2010), and therefore end up in the edible parts of the carcasses. This could be a potential problem from a food-safety viewpoint and for consumers in general

(Bergqvist et al., 2015). Also, there is a cost involved in the purchase and the placement of the microchips.

Besides thermochips, the use of non-contact and non-invasive methods like infrared thermography could potentially save time and reduce stress for the animals (Soerensen and Pedersen, 2015; Zhang et al., 2019). Lastly, a study by Siewert et al. (2014) suggested that infrared imaging may be used for early detection of elevated body temperature in pigs (> 39.5 °C).

5.2.2. Considerations and future research

While sensor technology and temperature implants offer promising advancements in monitoring diseases, some limitations need to be addressed. Firstly, factors such as housing conditions, management practices, and environmental stressors might significantly impact the accuracy and reliability of sensor data. Therefore, extrapolating findings from controlled environments to field conditions requires caution and further validation. Secondly, the limited sample size in our study may affect the extrapolation of the results observed for movement. Nevertheless, it gives an indication of its possible use in the near future. Thus, to ensure more reliable conclusions, studies with larger sample sizes are necessary to assess the variability between and within pig populations.

The short duration of the study may not have captured long-term trends, as chronic infections like *M. hyopneumoniae* may exhibit dynamic patterns over extended periods. Therefore, longitudinal studies are warranted to investigate the effectiveness of movement monitoring. Since movement sensors might be susceptible to technical issues, such as malfunction or data inaccuracies, as observed in our study, regular maintenance, updates, and validation protocols might be necessary to ensure data accuracy.

The upcoming era of smart sensors might have the potential to revolutionize disease monitoring in pig farming by providing continuous and real-time data on different health parameters, but validation in field conditions is essential to ensure the applicability and effectiveness of sensor technology in real-world pig farming settings. In addition, AI will most likely be trained to interpret the data originating from such devices, allowing the users to have applicable information at hand. Lastly, these upcoming technologies might

serve as supportive tools for farmers and veterinarians but will likely not replace the daily management of the farm nor the conventional detection tests (like PCR). The clinical expertise of the veterinarian will remain important.

5.3. General conclusions

Overall, the studies in this thesis explored the effects of infection with *M. hyopneumoniae* on pig respiratory health, providing insight into clinical, microbiological, and behavioral factors. Pig respiratory diseases are mostly associated with *M. hyopneumoniae*, where cranioventral pulmonary consolidation lesions are observed. In addition, lung lesions are often worsened by other pathogens. In the first study (Chapter 3), it can be inferred that pigs infected with *M. hyopneumoniae* had significantly less microbial diversity in their lungs, and NT samples exhibit more variation when compared to BALF samples, highlighting the importance of BALF sampling when investigating the LRT microbiome. In the second study (Chapter 4), the results indicate that *M. hyopneumoniae* infection alters the movement of pigs, in which infected animals move less than non-infected animals, suggesting that movement could be further investigated for early disease identification in pigs. Lastly, even though body temperature measurements did not correlate significantly with *M. hyopneumoniae* infection in our study, the results regarding its association with rectal temperature suggest that thermal microchips implanted intramuscularly might be useful to assess body temperature in pigs kept in experimental settings.

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7. CURRICULUM VITAE

Karina Sonalio was born in Arroio Trinta (Brazil) on the 5th of January 1993. She graduated as a veterinarian from the Faculty of Veterinary Medicine at the Federal University of Uberlândia in 2018, with the greatest distinction. In 2013/2014, during the undergraduate program, she participated in the Science Without Borders Program from Brazil having Indian Hills Community College (USA) and Iowa State University (USA) as host institutions. During that time, she followed some Animal Science courses and helped with research trials at the Veterinary Medical Research Institute. In 2017, before graduation, Karina worked as a supervisor trainee at BRF (a Brazilian food processing company) working at the swine slaughterhouse, in Uberlândia. In March 2018, she started her Master's degree in Veterinary Medicine with emphasis on swine health at São Paulo State University, Jaboticabal, where she worked on the genetic diversity of *Mycoplasma suis* and *Mycoplasma parvum*.

In 2020, Karina continued her studies and started her PhD on Veterinary Medicine and swine health at São Paulo State University (Jaboticabal), where she conducted the first study of this thesis, in which the respiratory microbiome of *M. hyopneumoniae*-infected and non-infected pigs was investigated. In 2021, the collaboration with the Porcine Health Management Team at Ghent University was established and she moved to Belgium to conduct part of her studies as a Joint PhD student. The studies she conducted at Ghent University focused on: i) the assessment of new tools for diagnostics of *M. hyopneumoniae*; ii) a new challenge model for *M. hyopneumoniae*; and iii) the efficacy of newly developed inactivated vaccines against *M. hyopneumoniae* (studies not included in this thesis).

Karina is the first author and co-author of several studies published in international peer reviewed journals and presented her research at different national and international conferences.

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