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The nasal microbiota under different scenarios associated with polyserositis in piglets

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PhD Thesis

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Tesis doctoral presentada por **Miguel Blanco Fuertes** para acceder al grado de Doctor en el marco del programa de Doctorado en *Medicina i Sanitat Animals de la Facultat de Veterinària de la Universitat Autònoma de Barcelona*, bajo la dirección de **Virginia Aragón Fernández**, **Marina Sibila Vidal** y **Florencia Correa Fiz**.

Bellaterra, 2023

La Dra. **Virginia Aragón Fernández**, la Dra. **Marina Sibila Vidal**, y la Dra. **Florencia Correa Fiz** investigadoras del *Institut de Recerca i Tecnologia Agroalimentàries - Centre de Recerca en Sanitat Animal* (IRTA-CReSA) y el Dr. **Joaquim Segalés Coma**, catedrático del *Departament de Sanitat i d'Anatomia Animals de la Facultat de Veterinària de la Universidad Autónoma de Barcelona*.

Certifican:

Que los trabajos de investigación desarrollados en la memoria de tesis doctoral "*The nasal microbiota under different scenarios associated with polyserositis in piglets*", presentados por el licenciado **Miguel Blanco Fuertes** para la obtención del Grado de Doctor en *Medicina i Sanitat Animals* se ha realizado bajo la dirección y tutoría, y autorizan su presentación a fin de ser evaluada por la comisión correspondiente.
Y porque así conste y tenga los efectos que correspondan, firman el presente certificado.

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Abbreviations

ADWG: average daily weight gain

AMR: antimicrobial resistance genes

ANCOM-BC: analysis of compositions of microbiomes with bias correction

ANCOM: analysis of compositions of microbiomes

ASV: amplicon sequence variant

BLAST: basic local alignment search tool

Bp: base pair

CEFT: ceftiofur

CFU: colonies forming unit

CNA: correlation network analysis

CPS: capsular polysaccharides

DNA: desoxyribonucleic acid

dNTPs: deoxynucleotide triphosphates

dTDP: deoxythymine diphosphate

ERIC: enterobacterial repetitive intergenic

FMT: fecal material transplantation

KEGG: Kyoto encyclopedia of genes and genomes

Log: logarithm

MAG: metagenome assembled genome

NAD: nicotinamide adenine dinucleotide

NCBI: national center for biotechnology information

OTU: operational taxonomic unit

PBS: phosphate buffer Solution

PC: principal component

PCA: principal component analysis

PCoA: principal coordinate analysis

PCR: Polymerase Chain Reaction

PERMANOVA: permutational multivariate analysis of variance

Qiime2: quantitative insights into microbial ecology

qPCR: quantitative polymerase chain reaction

RNA: ribonucleic acid

rRNA: ribosomic ribonucleic acid

SCNIC: sparse cooccurrence network investigation for compositional data

SRA: sequence read archive

STAMP: statistical analysis of microbial profiles

TCA: tricarboxylic acid cycle

WGS: whole genome shotgun

Abstract

Polyserositis is a common disease during the postweaning period of swine production, mainly caused by systemic invasion of the early colonizers of the upper respiratory tract *Glaesserella parasuis*, *Mycoplasma hyorhinis* or *Streptococcus suis*. Previous studies have demonstrated the role of the respiratory microbiota in the development of the polyserositis caused by *G. parasuis* (Glässer's disease), but the relation of the nasal microbiota with the other etiological agents of this disease has not been studied. In the present Thesis (Study I), the composition of the nasal microbiota of piglets from farms with recurrent problems of polyserositis associated with *M. hyorhinis* (MH) or Glässer's disease (GD) was analyzed and compared to piglets from healthy control farms (HC). Animals from MH or GD farms had a nasal microbiota with lower diversity than those from the HC farms. Interestingly, the composition of the nasal microbiota of piglets from MH farms was different from the one of animals from GD farms. These results suggested that divergent microbiota imbalances may predispose the animals to different systemic infections. In addition, the sequence variants of the pathogens associated with the corresponding disease were detected, highlighting the importance of studying the microbiome at strain-level resolution.

Antibiotic treatment is a strategy to control bacterial diseases, but it may have a negative impact on the microbiota. In Study II, piglets were followed from birth to 8 weeks of age (lactation to nursery) to assess the effect of ceftiofur on the pathogen's nasal colonization, as well as on the nasal microbiota composition, when the treatment was administered to pregnant sows or their newborn piglets. The administration of ceftiofur induced an unexpected temporary increase in alpha diversity at day 7 mainly due to colonization by environmental taxa. Moreover, this antibiotic treatment had a longer impact on the nasal microbiota of piglets when administered to the sows before farrowing than directly to them. This study also demonstrated, for the first time, the possibility of modifying the piglet nasal microbiota by inoculating, at birth, a pool of

nasal colonizers. The combination of sow treatment and colonization of piglets resulted in lower clinical signs during the nursery period. This can be probably explained by a reduction of pathogen transfer from the sows (due to the antibiotic treatment) and restoration of the altered microbiota by the beneficial colonizers.

Vaccination is another strategy to control diseases, including Glässer's disease. A third study investigated the effect of sow vaccination against virulent strains of *G. parasuis* on the nasal microbiota of their offspring. This study found that the composition of the nasal microbiota was different between piglets born to vaccinated or unvaccinated sows, with lower diversity in the vaccinated group and a strong clustering of the groups in beta diversity analysis. This study also reported that vaccination of the sows had a large effect on the microbiota composition of their offspring that went beyond the effect on the targeted pathogen. The mechanisms underneath these changes may include alteration of the microbiota network due to the elimination of the targeted pathogen and/or immunological changes.

Taken together, this dissertation highlights the importance of understanding the role of the nasal microbiota of piglets in the prevention and control of polyserositis disease in swine farming. The present Thesis also provides relevant insights into the impact of antibiotic treatment and sow vaccination on the piglet nasal microbiota and suggests potential strategies for microbiota interventions to improve animal health.

Resumen

La poliserositis es una enfermedad común en la producción porcina, principalmente tras el destete. Esta enfermedad está causada por la diseminación sistémica por los colonizadores tempranos del tracto respiratorio superior del cerdo, principalmente *Glaesserella parasuis*, *Mycoplasma hyorhinis* o *Streptococcus suis*. Estudios previos han demostrado el papel de la microbiota respiratoria en el desarrollo de la poliserositis causada por *G. parasuis* (enfermedad de Glässer), pero no se ha estudiado la relación de la microbiota nasal con los demás agentes etiológicos de esta enfermedad. En la presente Tesis (Estudio I), se analizó la composición de la microbiota nasal de lechones de granjas con problemas recurrentes de poliserositis asociada a *M. hyorhinis* (MH) o enfermedad de Glässer (GD) y se comparó con granjas sanas control (HC). Los animales de granjas HM o GD tenían una menor diversidad en la microbiota nasal que los de las granjas HC. Curiosmanete, la composición de la microbiota nasal de los lechones de las granjas MH fue diferente a la de los animales provenientes de las granjas GD. Estos resultados sugieren que distintos desequilibrios de la microbiota pueden predisponer a infecciones sistémicas diferentes. Además, se encontraron variantes de secuencias de los patógenos que se asociaron con la enfermedad correspondiente, destacando la importancia de estudiar el microbioma a nivel de cepa.

Los antibióticos se usan comúnmente para el control de las infecciones bacterianas, pero pueden tener un impacto negativo sobre la microbiota. En el Estudio II, un lote de lechones se siguió durante las primeras 8 semanas de vida para evaluar el efecto del ceftiofur en la colonización nasal de los patógenos, así como en la composición de la microbiota nasal, cuando el tratamiento se administró a cerdas o a sus camadas. La administración de ceftiofur indujo un aumento temporal inesperado en la diversidad alfa al día 7 debido principalmente a la colonización por taxones ambientales. Además, este tratamiento con antibióticos tuvo un impacto más prolongado cuando se administró a las cerdas que directamente a los lechones. Se demostró también, por

primera vez, la modificación de la microbiota nasal mediante la inoculación de colonizadores nasales al nacimiento. La combinación del tratamiento de las cerdas y la colonización de lechones resultó en menos signos clínicos durante la transición. Esto se podría explicar por una reducción en la transferencia de patógenos desde las cerdas (debida al tratamiento con antibióticos) y la restauración de la microbiota por los colonizadores.

La vacunación es otra estrategia para controlar las enfermedades infecciosas, incluida la enfermedad de Glässer. La vacunación de cerdas contra cepas virulentas de *G. parasuis* modificó la microbiota nasal de sus crías (Estudio III). La composición de la microbiota nasal del grupo vacunado mostró una menor diversidad y una fuerte divergencia respecto al grupo control en el análisis de diversidad beta. Este estudio también mostró que el efecto de la vacunación de las cerdas fue más allá de la reducción del patógeno frente al cual la vacuna iba dirigida. Los mecanismos subyacentes a estos cambios podrían incluir la alteración de la red de microbiota debido a la eliminación del patógeno y/o a cambios inmunológicos.

En conjunto, esta disertación destaca la importancia de comprender el papel de la microbiota nasal de los lechones en relación con la prevención y el control de la poliserositis en la cría de cerdos. La presente Tesis también proporciona información relevante sobre el impacto del tratamiento con antibióticos y la vacunación de las cerdas en la microbiota nasal de los lechones y sugiere intervenciones de la microbiota para mejorar la salud del animal.

Resum

La poliserositis és una malaltia freqüent de la producció porcina, principalment en el període post-deslletament, causada per la disseminació sistèmica dels colonitzadors primerencs nasals porcins, principalment *Glaesserella parasuis, Mycoplasma hyorhinis o Streptococcus suis.* Estudis previs han demostrat el paper de la microbiota respiratòria en el desenvolupament de poliserositis causades per *G. parasuis* (malaltia de Glässer), però no s'ha estudiat la relació microbiota nasal i altres agents etiològics de la malaltia. En la present Tesi (Estudi I), es va analitzar la composició de la microbiota nasal de garrins de granges amb problemes recurrents de poliserositis associada a M. *hyorhinis* (MH) o malaltia de Glässer (GD) i es va comparar amb granges sanes (HC). Els animals de les granges MH o GD tenien una microbiota nasal amb una diversitat més baixa que aquells de les HC. La composició de la microbiota nasal dels garrins de les granges MH va ser diferent dels de granges GD suggerint que diferents desequilibris de la microbiota poden predisposar a diferents infeccions sistèmiques. En l'estudi es van trobar variants de seqüències dels patògens associats a la corresponent malaltia, destacant la importància d'estudiar el microbioma amb resolució a nivell de soca.

El tractament amb antibiòtics és una estratègia per controlar les malalties bacterianes, però pot tenir un impacte negatiu en la microbiota. En l'Estudi II, es van seguir un lot de garrins fins a les 8 setmanes d'edat per tal d'avaluar l'efecte del ceftiofur en la colonització dels patògens nasals com en la composició de la microbiota nasal, quan el tractament es donava a les mares o als garrins. L'administració de ceftiofur va induir un increment inesperat i temporal de la diversitat alfa al dia 7 degut a la colonització per tàxons ambientals. També va tenir un impacte a la microbiota nasal dels garrins més durader quan va ser administrat a les mares gestants, que quan ho va ser directament a ells. Aquets estudi també va demostrar, la possibilitat de modificar la microbiota nasal del garrí inoculant al naixement, un grup de colonitzadors nasals. La combinació del tractament a les mares amb la colonització dels garrins

va reduir els signes clínics durant el període de la transició. Aquest fet es podria explicar per a la reducció de la transferència de patògens des de la mare i a la restauració de la microbiota per part dels colonitzadors.

La vacunació és una altre estratègia per controlar les malalties infeccioses, incloent la malaltia de Glässer. En un tercer estudi, es va investigar l'efecte en la microbiota nasal dels garrins de la vacunació de les truges en front a soques virulentes de *G. parasuis*. En aquest estudi, es va trobar que la composició de la microbiota nasal era diferent entre garrins nascuts de mares vacunades i els de no vacunades. També, el grup vacunat tenia una diversitat més baixa i una forta divergència envers al grup no vacunat en la diversitat beta. Aquest estudi també va descriure que la vacunació de mares tenia un efecte important en la microbiota dels garrins que anava més enllà del patògen d'interés. Els mecanismes darrera aquests canvis podrien incloure alteracions a la xarxa de la microbiota deguts a la eliminació dels patògen i/o canvis nivell immunològics.

En conjunt, aquesta dissertació destaca la importància de compendre el paper de la microbiota nasal dels garrins en relació a la prevenció i control de les poliserositis en la producció porcina. La present tesis també ofereix informació relevant sobre l'impacte del tractament antibiòtic i de la vacunació de les truges en la microbiota nasal dels garrins i suggereix potencials intervencions de la microbiota per millorar la salut dels animals.

GENERAL INTRODUCTION

1. Polyserositis in swine

1.1. Clinical signs and pathological observations

Polyserositis is a disease characterized by fibrinous or fibrinous-purulent exudates due to the inflammation of multiple serous membranes, including the pleura, pericardium, or peritoneum. The fibrinous exudate is usually accompanied by a variable amount of liquid. The inflammation and exudate formation can impair the function of the affected organs and lead to severe clinical disease. Clinical signs of polyserositis in pigs include fever, lethargy, anorexia, and respiratory distress (abdominal breeding). Affected pigs may also show joint swelling with lameness (with associated skin lesions) and nervous signs such as paddling and trembling. The disease can progress rapidly, leading to a high mortality rate in untreated pigs (Hattab et al., 2022a; Opriessnig et al., 2011; Salogni et al., 2020). Polyserositis is a common and devastating disease that affects pigs worldwide, frequently in the postweaning period, leading to substantial economic losses to the pig industry.

Polyserositis in young pigs is mainly caused by opportunistic pathogens that are common inhabitants of the upper respiratory tract: *Glaesserella parasuis*, *Mycoplasma hyorhinis*, and *Streptococcus suis* (Aragon et al., 2019; Gottschalk & Segura, 2019; Pieters & Maes, 2019). Diagnosis of polyserositis in pigs is based on clinical signs, postmortem findings, and laboratory tests. The definitive diagnosis of such clinical signs should be accompanied by the confirmation of the presence of the corresponding bacterium in the lesions. Differential diagnosis must include *Escherichia coli* as well.

1.2. Etiological agents

Glaesserella parasuis

Glaesserella parasuis is a rod-shaped Gram-negative bacterium that belongs to the family *Pasteurellaceae* and the class *Gammaproteobacteria* and is the causative agent of Glässer's disease; a severe and contagious disease in pigs (Aragon et al., 2019). The

bacterium was previously known as *Haemophilus parasuis*, but its classification changed in 2019 following a comprehensive taxonomic revision of the *Pasteurellaceae* family (Dickerman et al., 2020). The disease caused by *G. parasuis* causes a major economic concern for the swine industry worldwide due to its significant impact on pig health, welfare, and production (Costa-Hurtado et al., 2020).

The primary colonization of this bacterium takes place through contact with the sow after birth and it can be detected in the nasal cavity of piglets as early as 2 days of age (Cerdà-Cuéllar et al., 2010). Under a healthy scenario, colonization by *G. parasuis* develops when piglets are still protected by the maternal immunity acquired through colostrum and a balance between colonization and immunity is reached.

G. parasuis infection can range from acute to chronic forms. The most common clinical signs of the acute form of the disease include fever, anorexia, depression, respiratory distress, lameness, swollen joints, and sudden death. In contrast, the chronic form of the disease is characterized by milder clinical signs, such as coughing, labored breathing, and weight loss (Aragon et al., 2010, 2019). These clinical signs are nonspecific and can also be seen in other respiratory and systemic diseases in pigs, making it difficult to differentiate *G. parasuis* infection from other respiratory diseases based only on clinical signs (Palzer et al., 2015).

After colonization of the upper respiratory tract, virulent *G. parasuis* can reach the lung, where it survives from the action of the alveolar macrophages and disseminates (invasion) causing systemic disease and severe inflammation (Costa-Hurtado et al., 2012; Olvera et al., 2009; Vahle et al., 1995). More than one strain can be found per animal (in the nasal cavity) and in the same herd (Cerdà-Cuéllar et al., 2010). *G. parasuis* strains can present different degree of virulence (Aragon et al., 2010; Olvera et al., 2006). Serotyping has been classically proposed as the method to classify *G. parasuis* strains (Kielstein & Rapp-Gabrielson, 1992). More recently, studies seeking *G. parasuis* virulent factors discovered a group of virulence-associated trimeric autotransporters (VtaA), involved in phagocytosis resistance and adhesion to extracellular matrix protein (Costa-Hurtado et al., 2012, 2019). Specific PCRs targeting these virulent factors showed to be able to discriminate pathogenic and non-pathogenic strains (Galofré-Milà et

al., 2017a; Howell et al., 2014; Olvera et al., 2012). This family of VtaAs proteins and a specific fragment found only in the VtaAs from virulent strains were proposed to be used as a vaccine (Correa-Fiz et al., 2017; Olvera et al., 2012).

Mycoplasma hyorhinis

Mycoplasma hyorhinis is a small, cell-wall deficient bacterium that belongs to the family *Mycoplasmataceae* and to the class *Mollicutes* (Pieters & Maes, 2019). The pigs become infected by contact from either the sows or from infected pen mates (Pieters & Maes, 2019). While it is also a common inhabitant of the upper respiratory tract, *M. hyorhinis* has also been implicated in clinical diseases in nursery-age pigs, including polyarthritis, polyserositis and recently also meningitis (Bünger et al., 2020; Palzer et al., 2015; Pieters & Maes, 2019). The clinical signs associated to *M. hyorhinis* infection include, slight fever, depression, reduced appetite, reluctancy to move, difficulty to breathe, abdominal tenderness, swollen joints and lameness.

The pathogenesis of *M. hyorhinis* infection in pigs is still unknown, but it is thought that this bacterium can evade the host immune system by altering its surface antigens and by producing immunomodulatory molecules (Pieters & Maes, 2019). In addition, *M. hyorhinis* may induce a dysregulated immune response in the host, and can promote inflammation and tissue damage contributing to the development of polyserositis (Jayagopala Reddy et al., 2000; Trueeb et al., 2020).

The diagnosis of *M. hyorhinis*-associated disease in pigs can be challenging, as it requires the detection or the isolation of the bacterium in the samples taken from the affected body sites. The isolation of the bacteria requires some days to grow and needs to be confirmed by molecular methods (Clavijo et al., 2019). As in other colonizers, the detection of *M. hyorhinis* in lung, airways or upper respiratory tract is not confirmatory of systemic disease, as animals can be colonized by this commensal without showing clinical signs (Pieters and Maes 2019). A high genetic heterogeneity and variation of virulence among strains have been described for *M. hyorhinis* (Clavijo et al., 2019; J. Wang et al., 2022; Yamaguti et al., 2015). Although several virulence factors have

been suggested, up to now a virulence marker to differentiate between virulent and non-virulent strains has not been described.

Streptococcus suis

Streptococcus suis is a coccus-shaped Gram-positive bacterium that belongs to the *Streptococcaceae* family and the *Bacilli* order. *S. suis* is a significant pathogen for the swine industry where it causes significant economic losses (Gottschalk & Segura, 2019; Neila-Ibáñez et al., 2021). The piglets become naturally colonized at birth, when they go through the birth canal and later, through further contact with the sow. The bacterium colonizes the upper respiratory tract, preferentially the tonsils, of pigs, where it can be part of the healthy microbiota or spread throughout the body via the bloodstream (Gottschalk & Segura, 2019). *S. suis* is an emerging zoonotic agent that had increased in importance in the last 5 years (Wileman et al., 2019). Horizontal transmission is important during outbreaks when the shedding of the pathogen is higher in diseased animals (Cloutier et al., 2003). Lastly, aerosol transmission was demonstrated, at least in serotype 2, without direct nose contact (Berthelot-Hérault et al., 2001).

A wide range of clinical signs are described for this disease including fluctuating fever, nervous sings derived of the inflammation of the meninges (as incoordination, ataxia, paddling or convulsions), lameness, poor appetite, depression and anorexia. In peracute severe scenarios, pigs may be found dead without showing any previous signs (Gottschalk & Segura, 2019). Postmortem examination of diseased pigs typically reveals fibrinous exudates on the serous membranes, and pleural effusions. Microscopic examination of affected tissues may reveal inflammation, necrosis, and the presence of bacterial colonies. While the presumptive diagnosis can be done with the clinical signs and the postmortem findings, laboratory tests such as bacterial culture and PCR, are again needed to confirm the diagnosis of *S. suis* infection (Okura et al., 2014). In addition, the zoonotic potential of *S. suis* is a concern, as this bacterium can cause severe infections in humans, particularly in people who work in close contact with pigs (Lun et al., 2007).

The pathogenesis of *S. suis* in pigs is not fully understood, where the bacterium can cause a range of lesions, including meningitis,

arthritis, and septicemia. In polyserositis, *S. suis* infects the serous membranes of the body, leading to inflammation and fibrinous exudate formation. *S. suis* can express several virulence factors that contribute to its pathogenicity, including capsule, suilysin and muramidase-released protein (Tenenbaum et al., 2016). The prevalence of pig carrier rate is almost 100%, but the incidence of the disease is higher in piglets from 5 to 10 weeks of age (Cloutier et al., 2003).

S. suis has at least 35 different serotypes based on capsular polysaccharides (CPS), with serotypes 2 (serotype with zoonotic potential) and serotype 9 (most prevalent serotype in Spain) being the most commonly isolated from clinical cases of *S. suis* infections in Europe (Gottschalk & Segura, 2019). The relation between serotypes and virulence is not clear, but some serotypes are more likely detected in diseased animals while others are more commonly found in healthy piglets. As with *G. parasuis*, usually more than one *S. suis* serotype is present in the same animal but only the virulent strains are responsible for the disease (Gottschalk & Segura, 2019; Goyette-Desjardins et al., 2014). Several virulence factors have been proposed to differentiate virulent strains, specially from serotype 2 (Gottschalk & Segura, 2019).

1.3. Prevention and control

In general, prevention and control of polyserositis involve improved housing and management practices to reduce stress in the piglets. For instance, poor ventilation, and incorrect or fluctuating room temperature may be predisposing factors to the development of polyserositis in young pigs (Aragon et al., 2019; Gottschalk & Segura, 2019; Pieters & Maes, 2019).

The most widely used control measure used to treat polyserositis is the use of antimicrobials. Antibiotic treatment should be guided by susceptibility test. The main antimicrobials used for *G. parasuis* and *S. suis* are: synthetic penicillin, ceftiofur, ampicillin and enrofloxacin among others (Aragon et al., 2019). In the case of *M. hyorhinis,* tetracyclines, fluoroquinolones and macrolides are described as antimicrobial therapy to treat this disease (Klein et al., 2022).

Due to the elevated concern of antimicrobial resistances, which are a major problem in both human and animal health, finding alternatives to

the use of antibiotics is a priority nowadays. The use of perinatal antibiotics as prophylactic method was widely used to prevent polyserositis, but it can have a side effect eliminating beneficial commensal bacteria from the microbiota (Blaser, 2011). This was demonstrated in pigs when the perinatal antibiotics were removed from farms and the health was improved, as well as the diversity of the nasal microbiota, later in life (Correa-Fiz et al., 2019).

The main alternative to the use of antibiotics for controlling polyserositis is the use of vaccines. The commercial vaccines available for these pathogens are bacterins and show variable and inconsistent efficacy. Autogenous vaccines are also bacterins, and have been described for the three etiological agents (Corsaut et al., 2021; Dellagostin et al., 2023; Liu et al., 2016; Martelli et al., 2019). The selection of the strain and the adjuvant have a big impact on the performance of the vaccine (Hau et al., 2022; Martelli et al., 2019; McOrist, 2009; Obradovic et al., 2021). Experimental vaccines based on subunits (fragments) of virulent factors had been recently studied for *S. suis* and *G. parasuis* (Kralova et al., 2022; López-Serrano et al., 2021; Obradovic et al., 2021; Olvera et al., 2011), to provide cross-protection when the maternal immunity decreases in the postweaning period. Particularly, a fragment of the virulent factor VtaA in *G. parasuis* has been tested in sows showing an increased levels of specifics antibodies in their offspring (López-Serrano et al., 2021).

On the other hand, pathogen unspecific alternative to the use of antibiotics are probiotics: living microorganisms that given in the appropriate amount can confer a health benefit to the host. Probiotics constitute a holistic approach that involves the microbiota, remarking its importance in health and disease (Niederwerder, 2017).

2. The microbiota

2.1. Microbiota definition

The terms microbiota and microbiome are often used interchangeably, but they refer to different things (Berg et al., 2020). Microbiota refers to the assembly of living microorganisms from

different kingdoms (such as bacteria, fungi, and parasites) that inhabit a specific environment, such as the animal body. On the other hand, microbiome refers to the entire community of microorganisms and their genetic material, as well as the physical and chemical environment in which they live. The microbiome is the broader concept that encompasses the microbiota, along with all the other factors that influence the community of microbes in a particular environment (Berg et al., 2020; Su et al., 2012).

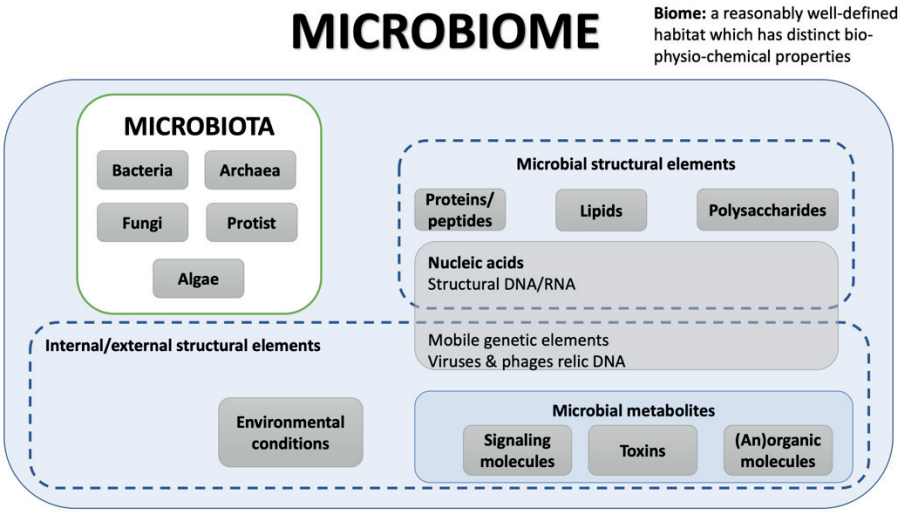

Figure 1. Scheme describing the composition of the term microbiome which contains the microbiota (community of microorganisms) and the rest of the elements (structural elements, metabolites/signal molecules, and the surrounding environmental conditions). Adapted from Berg et al. 2020.

2.2. Culture-independent methods to study the microbiota

With the exponential growth of high-throughput sequencing technologies, culture-independent approaches to study the microbiota have increased in the last decade (Pollock et al., 2018). The most common approaches include the marker gene sequencing and whole genome shotgun (WGS) metagenomics. The marker gene approach is the most extensively used consisting in the amplification and posterior sequencing of a marker gene. Marker genes are highly conserved genes that combine conserved domains with hypervariable regions, such as the 16S, 18S or ITS, being the 16S rRNA gene the one used for the

characterization of bacterial communities (Johnson et al., 2019). The combination of conserved and variable regions allows the taxonomic classification of the microorganisms by comparison with a curated database. The most used databases are Greengenes, SILVA, and Ribosomal Database Project (Cole et al., 2014; McDonald et al., 2012; Quast et al., 2013). On the other hand, with WGS metagenomics, there is no specific gene targeted and theoretically allows to sequence every DNA molecule in the sample. Both methods have strengths and limitations: the main bias of the marker gene approach is the need of a PCR amplification step prior to sequencing, which can introduce artifacts, duplicates, and chimeras that could deviate the composition of the data. Moreover, most of the 16S studies are based on the amplification of a small fragment of the 16S rRNA gene that not always has the resolution for taxonomic classification (Johnson et al., 2019). However, the success of the WGS approach relies on the possibility of sequencing the whole genetic material from the sample, and therefore explores additional aspects encoded in genome regions different than the 16S rRNA gene. Notwithstanding, the WGS method is not exempt from bias, since the DNA extraction method and the sequencing depth have more impact in this type of analysis than in the amplicon studies. In addition, the economic and computational costs, without mentioning the complexity of the downstream analyses, are higher in the WGS metagenomics compared with the marker gene studies.

3. The microbiota in pigs

The microbiome plays a crucial role in the health and performance of pigs. Among the ecological niches that conform the host microbiome, the most traditionally studied has been the gastrointestinal tract. Numerous studies have investigated the composition, diversity and stability of the swine microbiome across different developmental stages, production systems, and health conditions (H. B. Kim et al., 2011; Maltecca et al., 2021; Pollock et al., 2021; Wen et al., 2021). These studies showed that the composition and function of the gut microbiota was influenced by various factors, including environment (Megahed et al., 2019), diet (Yang et al., 2017), genetics (Bergamaschi et al., 2020)

and age (H. B. Kim et al., 2011). Understanding the swine microbiome and its interactions with the host can provide insights into improving animal health, welfare, and production efficiency (Bergamaschi et al., 2020; Ober et al., 2017).

As stated, one of the most important factors influencing the swine microbiome is age (Saladrigas-García et al., 2022). During the postnatal period, the piglet gut undergoes significant changes in anatomic structure and functionality, which impact the establishment and development of the microbiota. Studies have shown that the piglet gut microbiota is initially dominated by facultative anaerobes, such as *Enterobacteriaceae* and *Streptococcaceae*, and gradually shifts towards obligate anaerobes, such as *Mollicutes* and *Clostridiales*, as the piglet matures (Guevarra et al., 2019, 2021). The early-life microbiota plays a critical role in shaping the host immune system and gut physiology, which can have long-term effects on pig health and productivity (Maltecca et al., 2021; Saladrigas-García et al., 2022).

Another important factor influencing the swine gut microbiome is diet. The type and quantity of feed consumed by pigs can affect the nutrient availability, pH, and substrate diversity in the gut, which in turn influence the composition and activity of the microbiota (Pollock et al., 2021; Saladrigas-García et al., 2022). Studies have shown that dietary fiber, protein, and energy levels can modulate the swine gut microbiota and its metabolic output (D. Wang et al., 2020; Yang et al., 2017). The use of antibiotics in pig feed has also been shown to alter the microbiota composition and function, leading to concerns about the development of antibiotic resistance and the potential for negative impacts on animal health and the environment (Looft et al., 2012, 2014; Ortiz Sanjuán et al., 2022; Yang et al., 2017).

In addition to age, diet, and antimicrobial treatments, the swine microbiome can be influenced by various environmental factors, such as housing conditions, temperature, and humidity. Stressors such as heat stress, transport, and weaning can also affect the microbiota composition and diversity in pigs (Guevarra et al., 2019). Due to the plethora of factors involved, the study of the microbiota is not straightforward. Understanding the swine microbiome and its interactions with the host requires a multidisciplinary approach that combines microbiology, immunology, nutrition, and genetics. The use of high-throughput

sequencing technologies such as 16S rRNA gene sequencing, metagenomics, and metatranscriptomics has revolutionized our understanding of the swine microbiome and its role in host health and productivity. These approaches enable the comprehensive characterization of microbial diversity, function, and interactions in the gut ecosystem, providing insights into potential therapeutic and management strategies to improve pig health and production efficiency.

4. Respiratory microbiota in pigs

Most of microbiome studies in swine have mainly targeted the gut microbiota but the number of studies focusing on other niches, such as tonsils, skin, and nasal cavity, is increasing each day (Correa-Fiz et al., 2016; Fredriksen, Guan, et al., 2022; Strube et al., 2018; Q. Wang et al., 2018). The swine respiratory microbiota is a complex ecosystem composed of a diverse group of microorganisms that inhabit their respiratory tract. The nasal cavity is primarily inhabited by bacteria from the phyla *Firmicutes*, *Actinobacteria*, *Proteobacteria*, and *Bacteroidetes*. The most abundant bacterial families in the swine respiratory microbiota are *Pasteurellaceae*, *Streptococcaceae*, and *Mycoplasmataceae* (Pirolo et al., 2021).

As happens in other ecological niches, microorganisms in the respiratory tract can be affected by a plethora of external factors, but the high environmental exposure of the pig nostrils remarks the importance to study this ecological niche.

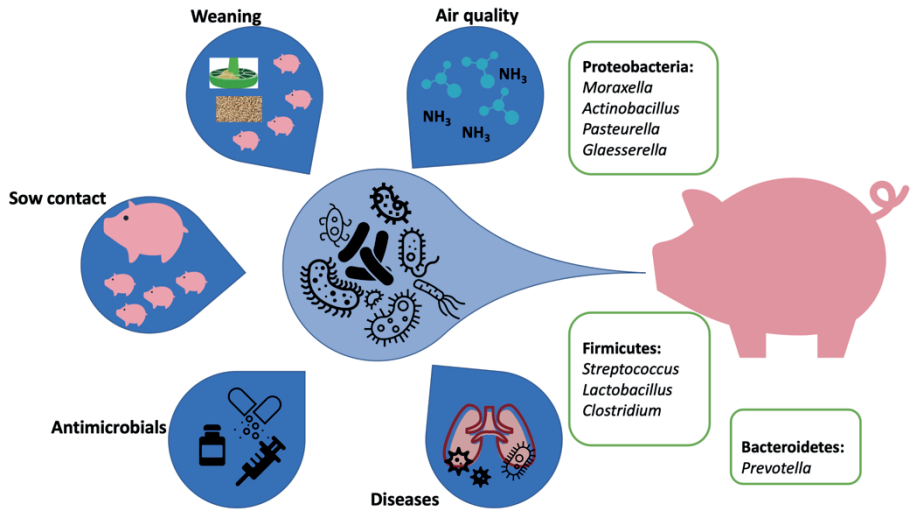

Figure 2. Bacterial composition and factors shaping the porcine respiratory microbiome. The porcine upper respiratory tract is mainly colonized by members of the *Proteobacteria*, *Firmicutes*, and *Bacteroidetes*. Several factors, including respiratory disease onset, farm management practices and antimicrobial treatment, contribute to the reduction of the bacterial diversity. Adapted from Pirolo et al., 2021

The first study of the porcine nasal microbiota was done in slaughter-age pigs, where Weese et al. evaluated the impact of the nasal microbiota on the methicillin-resistant *Staphylococcus aureus* (MRSA) presence in the nasal cavity with no significant results (Weese et al., 2014). On the contrary, Espinosa-Gongora et al (2016) found several taxa that were significantly associated to MRSA non-carriers, including lactic acid-producing species with potential probiotic and antimicrobial action (Espinosa-Gongora 2016). Moreover, they identified some pathogens, such as *Pasteurella multocida* and *Klebsiella*, that were associated with MRSA carriers. These discrepancies between the two studies may be associated to the phenotype classification criteria, laboratory and bioinformatic analysis. The studies mentioned were performed in slaughter-age pigs, but as previously shown for gut, the microbiota composition is known to be dynamic. Accordingly, a longitudinal study described the nasal microbiota during the first 7 weeks of age comparing with the fecal microbiota over time (Slifierz et al., 2015). Both microbiotas showed increasing diversity and richness with age until two weeks post-weaning, when they acquired a certain degree of stability (Slifierz et al., 2015).

The first publication showing the nasal microbiota composition and diversity at weaning as a predisposing factor to develop respiratory disease focused on Glässer's disease (Correa-Fiz et al., 2016). In this study, the nasal microbiota was characterized in weaning piglets from farms with different health status and different countries, showing that these factors affect the nasal microbiota at weaning.

5. Microbiota in Health and Disease

The study of the microbiota as a dynamic set of microbial communities that changes under different conditions is key to understanding its role in health and disease. Undoubtedly, one of the fundamental applications where microbiota studies are being applied is in the establishment of similarities and differences in the microbiota composition between individuals with different health status (Bass et al., 2019; Cho & Blaser, 2012; Hou et al., 2022; Natalini et al., 2023). This association between disease and microbiota has been extensively studied in gut microbiota in recent years. The relationship between respiratory infections and the airway microbiota has been also studied in animals (Mach et al., 2021) including pigs (Niederwerder, 2017; Ober et al., 2017). Furthermore, infectious viral diseases such as porcine reproductive and respiratory syndrome virus (PRRSV) and porcine circovirus type 2 (PCV2) were shown to impact the swine fecal microbiome and host health (Argüello et al., 2021; Niederwerder, 2017; Ober et al., 2017).

The respiratory microbiota has also been studied in relation to diseases caused by common inhabitants of the upper respiratory tract (Correa-Fiz et al., 2016). Correa-Fiz et al. (2016) not only characterized for the first time the nasal microbiota of the weaning piglets from different farms, but also compared the nasal microbiota of piglets from farms with a good health status against the nasal microbiota from farms associated with Glässer's disease. Among other results, Correa-Fiz et al. (2016) observed a lower alpha diversity and richness in the piglets from diseased farms and an increment in OTUs classified as *Glaesserella* in the disease-associated farms. Higher relative abundance of the phyla *Bacteroidetes*, *Proteobacteria* and *Tenericutes* was also associated with

Glässer's disease, showing that the nasal microbiota of animals from farms with recurrent problems of Glässer's disease was altered before the onset of the clinical signs, suggesting for the first time, that this can be a predisposing factor of the subsequent development of disease.
The cornerstone of these studies is to extract common trends and patterns in microbial composition associated to health. In addition, these analyses open the door for future research in data-driven science and predictive approaches.

6. Microbiota interventions

Microbiota intervention refers to the process of deliberately altering the composition or activity of the microbial communities that inhabit the host. These interventions can be done through a variety of methods, including dietary changes, probiotics, prebiotics, antibiotics, fecal microbiota transplantation (FMT), and other emerging therapies (Marrs & Walter, 2021; Suez et al., 2019). These methods are nowadays being developed to improve the host health in aspects as diverse as infectious disease (Lawley et al., 2012), productivity (Foo et al., 2017), and conservation (Jin Song et al., 2019).

Probiotics are defined by the Food and Agricultural Organization (FAO) and the World Health Administration (WHO) as "live microorganism that when administered in adequate amounts confer a health benefit on the host". The use of probiotics to modulate the respiratory microbiota and prevent respiratory diseases has been investigated in several studies in human health (Aimbire et al., 2019; Gelardi et al., 2020; Hao et al., 2015). Most of the probiotic studies for respiratory diseases use oral delivery as the route for administration. For instance, the oral administration of probiotics (pool of different species of *Lactobacillus*, *Bifidobacterium* and one *Streptococcus thermophilus*) was studied in the treatment of asthma, reducing the risk of asthma exacerbation, and improving asthma control compared to placebo or no intervention. In another study, oral probiotics were also investigated for their potential to prevent or reduce the severity of chronic obstructive pulmonary disease, where they found that a combination of three species from the genus *Lactobacillus* significantly improved lung function and quality of life compared to a placebo after 12 weeks of treatment (Aimbire et al., 2019). Recently, intranasal administration had been also

used for treating the asthma in mice with positive results (Spacova et al., 2019) and for chronic rhinosinusitis, where the intranasal administration of Lactococcus improved sinus-specific symptom (Al-Romaih et al., 2023).

The use of probiotics in pigs has been tested mostly with the objective to improve intestinal health and prevent gastrointestinal disorders (Vasquez et al., 2022). However, the re-establishment of the microbiota by probiotics after infection has not been demonstrated in domestic animals (Mach et al., 2021). These strategies have not been tested in respiratory diseases yet.

HYPOTHESIS AND OBJECTIVES

At the time this thesis was starting, only a previous study exploring the relationship of the swine nasal microbiota and disease was available. In this study, it was shown that the piglets' nasal microbiota composition can be a predisposing factor for Glässer's disease; a systemic infection caused by the early colonizer of the upper respiratory tract, *Glaesserella parasuis* (Correa-Fiz et al., 2016). Bacterial diseases in pigs can be controlled by antimicrobial therapy and/or vaccination, but due to low-efficient or lack of specific vaccines together with the worldwide increased antimicrobial resistances that forced to reduce their usage, alternative strategies to help control and prevention of diseases in pig farms are mandatory. The **hypothesis** is that the nasal microbiota composition plays an important role, not only in Glässer's disease, but also in other diseases of piglets of respiratory origin. Following this rational, we also hypothesize that the modulation of the nasal microbiota composition with the colonization by beneficial bacteria can help to control these problems. In this thesis, the interrelation among respiratory health, nasal microbiota, and the different tools used to control bacterial respiratory diseases are explored.

Objectives:

The general objective of this thesis is to extend the knowledge on the role of the nasal microbiota in polyserositis-associated diseases and understand whether treatment and prevention measures against these diseases, such as the use of antibiotics and vaccination, may affect the composition of the nasal cavity microbiome. Moreover, this thesis aimed also to assess the use of beneficial bacteria of the upper respiratory tract as an alternative to antibiotics to control systemic diseases caused by early colonizers of the upper respiratory tract.

The **specific objectives** are:

1. To evaluate the role of the nasal microbiota in the development of polyserositis by *Mycoplasma hyorhinis*.

2. To compare the changes of the nasal microbiota in piglets predisposed to suffer Glässer's disease or *M. hyorhinis* systemic disease.

3. To determine the effect of the antimicrobial treatment in sow and/or piglet in the composition of the piglet nasal microbiota.

4. To explore the possibility of modulating the piglet nasal microbiota through the inoculation of putative beneficial colonizers (probiotic candidates).

5. To determine the effect of sow vaccination against virulent strains of *Glaesserella parasuis* in the nasal microbiota of their offspring.

STUDY I

Altered nasal microbiota composition associated with development of polyserositis by *Mycoplasma hyorhinis*

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ABSTRACT

The abstract Fibrinous polyserositis in swine farming is a common pathological finding in nursery animals. The differential diagnosis of this finding should include Glaesserella parasuis (aetiological agent of Glässer's disease) and *Mycoplasma hyorhinis*, among others. These microorganisms are early colonizers of the upper respiratory tract of piglets. The composition of the nasal microbiota at weaning was shown to constitute a predisposing factor for the development of Glässer's disease. Here, we unravel the role of the nasal microbiota in the subsequent systemic infection by *M. hyorhinis*, and the similarities and differences with Glässer's disease. Nasal samples from farms with recurrent problems with polyserositis associated with *M. hyorhinis* (MH) or Glässer's disease (GD) were included in this study, together with healthy control farms (HC). Nasal swabs were taken from piglets in MH farms at weaning, before the onset of the clinical outbreaks, and were submitted to 16S rRNA gene amplicon sequencing (V3–V4 region). These sequences were analyzed together with sequences from similar samples previously obtained in GD and HC farms. Animals from farms with disease (MH and GD) had a nasal microbiota with lower diversity than those from the HC farms. However, the composition of the nasal microbiota of the piglets from these disease farms was different, suggesting that divergent microbiota imbalances may predispose the animals to the two systemic infections. We also found variants of the pathogens that were associated with the farms with the corresponding disease, highlighting the importance of studying the microbiome at strain-level resolution.

Keywords: porcine polyserositis; nasal microbiota; *Mycoplasma hyorhinis*; microbial diversity; 16S rRNA gene; Glässer's disease.

INTRODUCTION

The microbiota is defined as the community of microorganisms found in the Surface of a tissue, which represents their usual ecological niche (NIH HMP Working Group et al., 2009). Several studies have found an association between the microbiota composition and phenotypic features from the host, such as body weight, health status and disease onset, among others (D. Zheng et al., 2020). In animals, and in particular swine, the gut microbiota has been deeply studied in contrast to other body sites. The porcine intestinal microbiota influences several production parameters like body weight (Han et al., 2017) and feed efficiency (McCormack et al., 2017). The alterations in its composition can affect the translocation of metabolites across a disrupted intestinal barrier, which in turn could promote metabolic pathologies in various organs, such as liver and adipose tissue (McCormack et al., 2017). Although to a lesser extent, the nasal microbiota has also been associated with the development of diseases (Man et al., 2017). In swine, the nasal microbiota has been suggested as a predisposing factor for the development of Glässer's disease, a systemic infection caused by *Glaesserella* (*Haemophilus*) *parasuis* and characterized by fibrinous polyserositis (Correa-Fiz et al., 2016). Polyserositis is a common finding in necropsies during the post-weaning period. This pathology is not only caused by G. parasuis, but also by other bacteria, such as *Mycoplasma hyorhinis* (Pieters & Maes, 2019). These microorganisms are considered early colonizers of the respiratory tract, as they are part of the bacterial communities that conform the normal microbiota of the nasal cavity of piglets early in life (Correa-Fiz, Fraile, and Aragon 2016; Clavijo et al. 2019; Roos et al. 2019; Cerdà-Cuéllar et al. 2010). How these etiological agents switch from members of the normal microbiota to disseminate and induce systemic infection is the key to understand the pathogenesis of these diseases. A balance between colonization by these potential pathogens and the host is established during the early stages of the piglets' life (Costa-Hurtado et al., 2020) where the immune system plays a crucial role to establish this balance and maintain health. Recently, the role of particular taxa from the piglet microbiota has been shown in the polyserositis caused by *G. parasuis* where Lactobacillus and *Prevotella* were associated with health, while *Moraxella*, *Haemophilus*

(*Glaesserella*) and *Streptococcus* were associated with Glässer's disease (Correa-Fiz et al., 2016; Mahmmod et al., 2020). Similarly, differences in the oropharyngeal microbiota have been associated with the development of respiratory diseases in pigs (Q. Wang et al., 2018). Higher relative abundance of the *Moraxella* genus was associated with respiratory pathology and Lactobacillus was associated with healthy animals (Valeris-Chacin et al., 2021; Q. Wang et al., 2018). Here, we compare the piglet nasal microbiota composition at weaning, in farms with and without polyserositis cases caused by *M. hyorhinis*. Furthermore, we compare the nasal microbiota composition from a *M. hyorhinis*-affected farm, with data from previously characterized farms with recurrent outbreaks of Glässer's disease, and divergent changes from health between both pathological scenarios.

RESULTS

Alpha Diversity Is Significantly Reduced in the Nasal Microbiota of Piglets from Farms with Polyserositis

Farms with post-weaning polyserositis caused by *M. hyorhinis* (MH group) or *G. parasuis* (GD group), together with healthy control (HC group) farms, were included in this study (Table 1). Nasal samples were obtained from piglets at weaning and the nasal microbiota was determined by 16S rRNA gene sequencing. We obtained a total of 33,012,373 reads from the sequencing data of 74 nasal samples. The minimum number of reads in a sample was 70,970 and the maximum was 121,478. After quality-control, denoising and chimera removal, a total of 5,061,172 sequences were included in the posterior analysis.

Table 1 Main characteristics of the farms and number of samples included in the study

Farm (n*)	Group	Health status	Production system	Size**	Treatments***	Reference
RM (10)	MH	Polyserositis by *M. hyorhinis*	Multi-site	650	Amx	This study
GE (10)	MH	Polyserositis by *M. hyorhinis*	Multi-site	800	Amx	This study
VL (5)	HC	Healthy	Farrow to finish	700	Tlt-Ceft	[6]
PT (5)	HC	Healthy	Multi-site	1000	NA	[6]
GM (10)	HC	Healthy	Multi-site	1200	Amx	[6]
MT (8)	GD	Glässer's disease	Multi-site	3300	Pen-Strep	[6]
MC (10)	GD	Glässer's disease	Farrow to finish	480	Ceft	[6]
RC (6)	GD	Glässer's disease	Multi-site	1400	Ceft	[6]
EJ (10)	GD	Glässer's disease	Multi-site	2000	Enro	[6]

The feature table was rarefied to 6,545 sequences per sample and alpha diversity was estimated using different metrics. Microbial richness was assessed by calculating the observed features, which gives the count of the different amplicon sequence variants (ASVs) in rarefied samples. We found a total of 25,993 ASVs, with significant differences in richness among the three farm groups (Kruskal-Wallis; $p = 0.027$). Richness was significantly higher in the HC farms when compared with the farms with polyserositis MH and GD individually (Figure 1A). Chao index showed the same trend and detected the same differences between the groups (not shown). Alpha diversity was additionally assessed by Shannon index (Figure 1B), which considers both the richness and the evenness of the samples, and also detected differences among the three groups (Kruskal-

Wallis; p = 0.017). Farms with disease showed lower diversity than HC group when compared individually (HC vs MH and HC vs GD, p = 0.01 in both cases; Figure 1B)

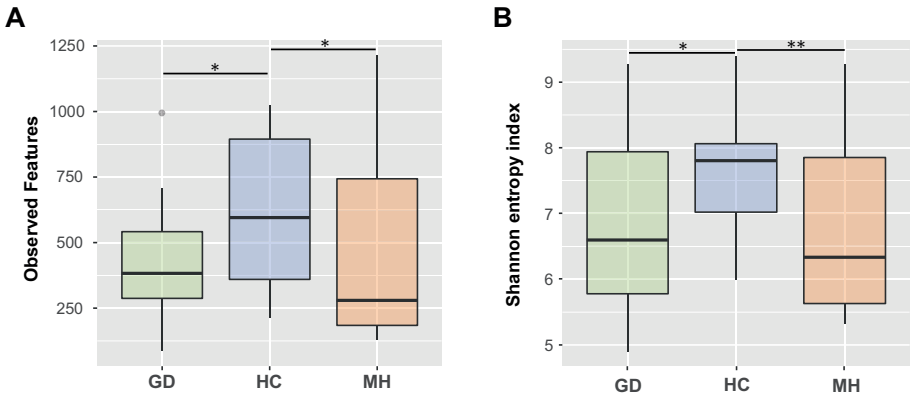

Figure 1 Boxplots representing median and interquartile ranges of alpha diversity estimated measuring the observed features (A) or Shannon's index (B) in nasal microbiota from piglets at weaning from healthy control farms (HC) and farms with polyserositis in the nursery caused by *Mycoplasma hyorhinis* (MH) or caused by *Glaesserella parasuis* (Glässer's disease, GD). Outlier is indicated with a grey circle on the plot. Error bars are standard deviation. * means $p < 0.05$; ** means $p \leq 0.01$.

Piglets from Farms with Polyserositis by *M. hyorhinis* Showed Significatively Different Nasal Microbiota Composition Compared to Piglets from HC and GD Farms

The differences in composition (beta diversity) of the nasal microbial communities among the different study groups were explored using Principal Coordinates Analysis (PCoA). The beta diversity analysis was done using Bray Curtis distance to measure the compositional dissimilarity quantitatively between different groups. The PCoA plot showed a more similar nasal microbiota composition between animals from GD and HC farms compared with the MH farms (Figure 2A). Although strong clustering was observed when the samples were compared considering the farm of origin (R2 = 47% estimated by Adonis function), the differences among the three groups of farms were explained by 17.3% (R2, p = 0.001, 999 permutations). Moreover, to understand the qualitative dissimilarities between groups the Jaccard distances were calculated. The Adonis function on this qualitative

approach retrieved values for R2 of 26.12% and 9.52% for farm or group clustering, respectively (Supplementary Figure S1A).

We also measured the qualitative (unweighted) and quantitative (weighted) measures of community dissimilarity incorporating the phylogenetic relationships between features (Unifrac). The percentage explained by the clustering of these groups was higher in the unweighted analysis (R2 = 12.07% Adonis, p = 0.01; Figure 2B) than in the weighted analysis (R2 = 10.04% Adonis, p < 0.01; Supplementary Figure S1B). The farm of origin had also a strong effect in the clustering (R2 = 36.6% Adonis, p = 0.01).

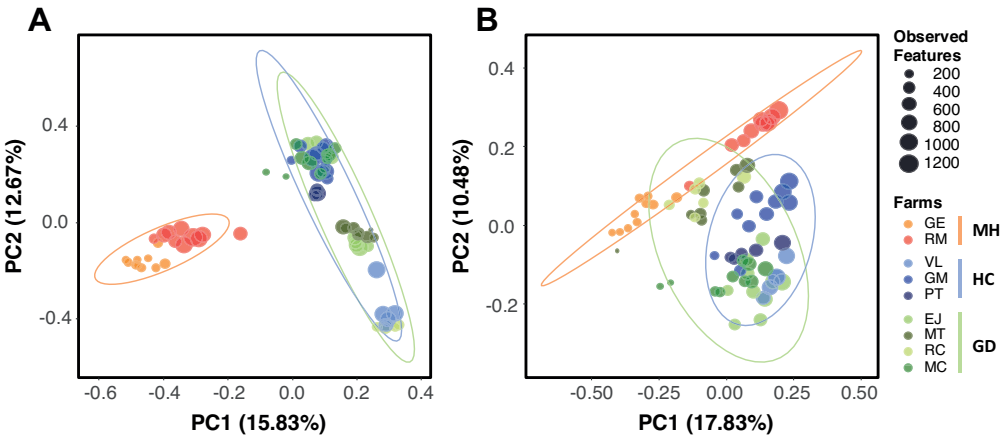

Figure 2 Beta diversity analysis on Bray Curtis (A) and unweighted Unifrac distances (B) of the nasal microbiota of weaning piglets. Each circle represents the microbial composition of each sample and the size of the circles is proportional to the number of Observed Features as indicated in the legend. Healthy control farms (HC) are depicted in blue, while farms with polyserositis caused by *M. hyorhinis* (MH) are in orange and *G. parasuis* (GD) in green palettes. Ellipses are calculated with the euclidean distances of the grouped samples.

Taxonomic Assignment of the ASVs

Taxa assignment was done by Naive Bayes classification algorithm using the Greengenes 13.8 16S gene database. Taxonomy was assigned to a total of 25,993 ASVs. The percentage of unassigned ASVs was increasing from phylum, with 0.96% of unassigned taxa, to lower taxonomic levels until species where 89.44% were found.

At phylum level, more than the 80% of the relative abundance were shared between three phyla, Proteobacteria, Bacteroidetes and Firmicutes. Proteobacteria was the most relative abundant phylum in the disease groups, while Bacteroidetes and Firmicutes were the most relatively abundant in the healthy group. *Gammaproteobacteria* was the most relative abundant bacterial class of the microbiota composition in the disease groups (MH and GD), while *Clostridia* was the most abundant in the healthy one. Indeed, *Clostridiales* and *Pseudomonales* were the most represented orders in the HC and disease groups, respectively.

At the family level, the most abundant were *Moraxellaceae*, *Weeksellaceae*, *Pasteurellaceae*, *Ruminococcaceae*, *Lachnospiraceae*, *Prevotellaceae*, *Muribaculaceae* (S24-7), *Streptococcaceae* and *Mycoplasmataceae*. *Moraxellaceae* was the most relative abundant family in all groups, with 17.69% in the HC group, 26.09% in GD and 32.30% in the MH group.

The most abundant genus in all groups was *Bergeyella*, with 14.45% in the HC group, 17.26% in GD and 20.85% in the MH group. *Enhydrobacter* was the second most abundant genus in HC and MH group with 6.30% and 18.19%, respectively. *Moraxella* was the second most abundant genus in GD (9.73%) and the third one in the MH group with the same percentage of 9.73. Finally, an unclassified genus from *Moraxellaceae* was the third most abundant in HC (5.76%) and GD (9.39%).

The 30 most abundant ASVs across all the samples belonged to *Moraxellaceae* (n = 4), *Moraxella* (n = 2), *Enhydrobacter* (n = 7), *G.* (*Haemophilus*) *parasuis* (n = 1) and *Bergeyella zoohelcum* (n = 17), which were distinctly distributed depending on the group (Figure 3). If we focus on the ASVs from *G. parasuis* and *M. hyorhinis*, the aethiological agents of the polyserositis observed in the farms, 45 ASVs were classified as *M. hyorhinis* (Figure 4A) and 42 as *G. parasuis* (Figure 4B). Curiously, the ASVs from these pathogens were not shared among the groups, but they were shared among different farms from the same group (Figure 4C,D). Interestingly, the amplicon 16S sequences

amplified from the isolated clinical samples were highly similar (99–100%) to the two most abundant *M. hyorhinis* ASVs in the MH group clustering together into the phylogenetic tree (Figure 4A, strong yellow).

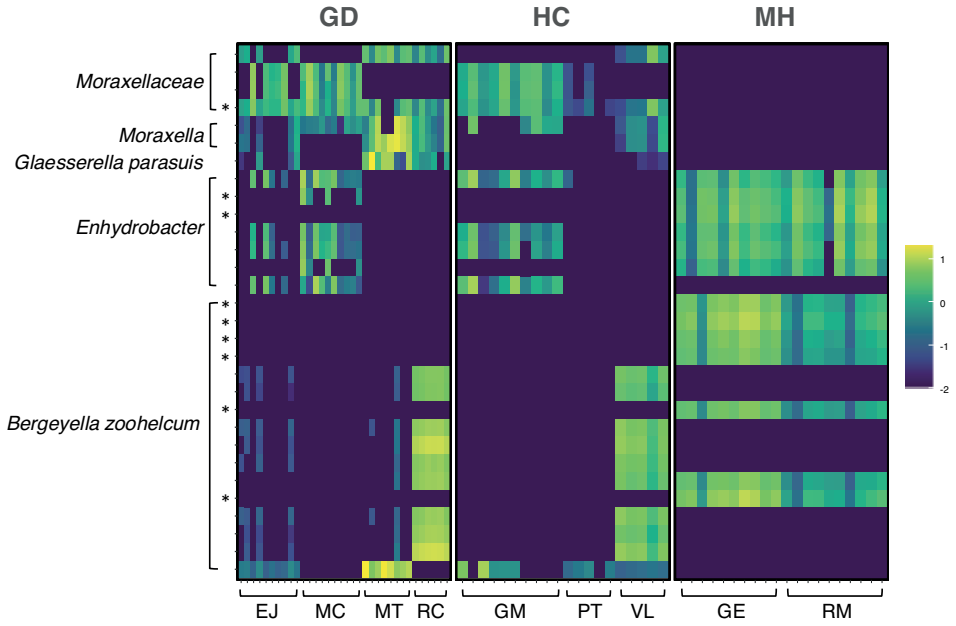

Figure 3. Relative abundance in the nasal microbiota of weaning piglets of the top 30 most abundant ASVs among all the groups in log10 scale. The X axis (bottom) shows the distribution of each sample by farm and the Y axis represents the top 30 most abundant ASVs grouped by the taxa assignment. Top labels correspond to the study groups: Glässer's disease (GD), healthy control (HC) and *M. hyorhinis* (MH) farms. Asterisks mark the ASVs statistically different in the differential abundance analysis (ANCOM) among the three study groups (GD, MH, HC).

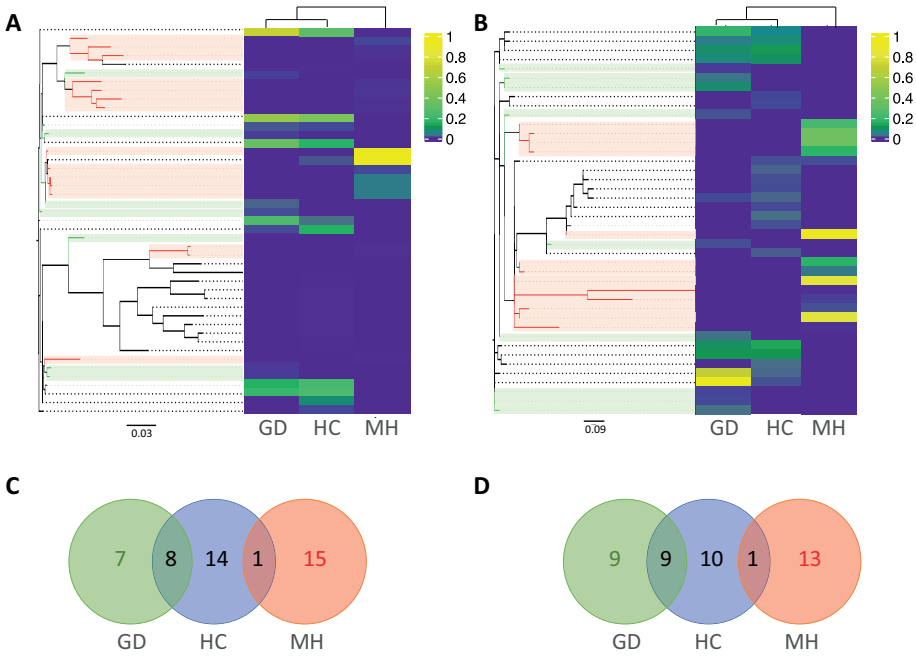

Figure 4. Relative abundance of *M. hyorhinis* (A) and G. parasuis (B) amplicon sequence variants (ASVs) in the nasal microbiota of weaning piglets from farms with Glässer's disease (GD), healthy controls (HC) or farms with polyserositis caused by *M. hyorhinis* (MH). The phylogenetic relationship of ASVs from each pathogen is represented in Maximum likelihood trees. Branches from ASVs found exclusively in GD farms are colored in green while the ones found only the MH group are colored in red. Venn diagrams representing the distribution of *M. hyorhinis* (C) and *G. parasuis* (D) ASVs in the different groups were plotted following the same color pattern.

Differential abundance analysis

To find out taxa differentially abundant among different groups we used the Analysis of composition of Microbiome (ANCOM) method. When the nasal composition from the three groups was compared, 144 differential ASVs were detected (Supplementary Table S7). The most significant ASVs belonged to *Moraxellaceae*, *Bergeyella zoohelcum*, *Enhydrobacter*, *Moraxella* and *Rothia nasimurium*. Remarkably, most of these ASVs detected as differential were present with a high relative abundance in the MH group when compared with the other two groups. Two of the high-abundant ASVs from MH group were classified as *M. hyorhinis*, depicted in Figure 4A in bright yellow. On the other hand, one

ASV was classified as *G. parasuis* but was only present in the MH group. Moreover, nine of the differentially abundant ASVs were found among the top 30 most abundant among all the groups globally, which are indicated with an asterisk in Figure 3.

To explore if there were ASVs associated to the health status, we analyzed groups MH and GD joined as a "disease group", against control (HC). Only eight features appeared as differentially abundant between these two groups, including three from *Prevotella* genus and one from *Streptococcus alactolyticus*, all in higher abundance in healthy farms (Supplementary Table S1). Additionally, when GD group was compared to the HC, only one ASVs from the *Lachnospiraceae* family was detected as differentially abundant. However, when MH was compared to HC, 67 ASVs were detected (Supplementary Table S1), where the ten most significant ASVs included seven classified as *B. zoohelcum*, two as *Enhydrobacter* and one as *Moraxellaceae* family. These latter ASV was the only one (from these ten) associated with the control farms (absent in MH farms), since the seven ASVs from *B. zoohelcum* were only present in the MH group, (with relative abundances ranging from 1.73% to 3.51%) and the two ASVs classified as *Enhydrobacter* were absent in HC farms (Supplementary Table S1).

The differences observed at the ASVs level were not reflected in the differential analysis (ANCOM) derived from the collapsed ASVs table at the different taxonomic levels (Supplementary Tables S2–S7).

DISCUSSION

Culture-independent studies for interpreting the swine microbiome in disease infections can be challenging, especially if the study aims to elucidate the differences in the microbiome before the onset of the clinical signs as a predisposing factor. Here, we studied the relationship between the nasal microbiota and the later development of the systemic infection by the early colonizers of the respiratory tract, *M. hyorhinis* and *G. parasuis*. We found lower alpha diversity in disease farms, with the nasal microbiota from piglets from farms with disease by *M. hyorhinis* showing higher divergence from the healthy farms than those with Glässer's disease. As it has been previously reported,

imbalances in richness and/or diversity in different microbiome landscapes are associated with changes that generally may lead to disease in the host organism (Ojetti et al., 2009). In swine production, changes in the nasal microbiota of the weaning piglets may influence the subsequent development of Glässer's disease (Correa-Fiz et al., 2016).

Antimicrobial usage is one of the plethora of factors that can affect the pig microbiome in commercial farms. The animals included in this study underwent different antimicrobial treatments, which may impact the nasal microbiota composition. However, we found that different farms under the same antimicrobial treatment but with different health status (GE, RM, GM) did not cluster together, suggesting that these treatments did not seem to be determinant in the subsequent health status of the animals. Although the present study presents some limitations due to the different factors affecting the microbiome composition of the animals in commercial farms, our results reinforce the general idea that lower alpha diversity is related with disease development (Correa-Fiz et al., 2016; Prehn-Kristensen et al., 2018). Lower bacterial richness and evenness was found in the nasal microbiota in the farms with disease, when they were compared with the animals from the healthy control farms. This finding is supported by many other studies that established that lower alpha diversity values are linked to poor health status in pigs, but also in other species (Pirolo et al., 2021; Prehn-Kristensen et al., 2018; Q. Wang et al., 2018). Here, a lower microbial richness of the nasal cavity in the disease farms may be associated with the proliferation of the undesirable taxonomic groups that would lead to systemic infections. However, the nasal microbiota composition from the two different disease groups differed, which indicates that different microbiota imbalances may predispose the animals to different infections. In general, the composition from the farms with Glässer's disease were more similar to the healthy ones but different from the *M. hyorhinis* farms. At higher taxonomic levels (phylum to order), highly similar composition between the disease groups MH and GD was observed, while at lower levels (family to species) differences between these two disease groups were evident. In fact, the distribution of the most abundant ASVs showed a clear different pattern in MH and GD farms. Nevertheless, we were able to find some taxa associated with health, such as the *Prevotellaceae* family, which

deserves further study. This family was also represented in the healthy farms at the ASVs level by *Prevotella copri* and *Prevotella stercorea*, two species previously found in higher abundance in suckling and weaning healthy piglets (Amat et al., 2020a). Two ASVs from the *Lachnospiraceae* family were also associated with healthy farms, in agreement with previous studies, where ASVs from this family were correlated with higher feed conversion ratio (Quan et al., 2018).

Animals from control farms showed a lower relative abundance of *M. hyorhinis* and *G. parasuis*. However, the relative abundance of the pathogens at the species level was not related to the posterior development of the corresponding systemic disease. Surprisingly, *G. parasuis* abundance was higher in MH farms, while *M. hyorhinis* was higher in GD farms. Although we cannot rule out a synergistic association between these two pathogens to develop disease, as it has been described before (Palzer et al., 2015), our data seems to support the role of specific ASVs of each pathogen in disease development. It is well known that *G. parasuis* comprises strains of different pathogenic potential with different consequences for the piglet health (Brockmeier et al., 2013; Galofré-Milà et al., 2017b). In fact, colonization by virulent *G. parasuis* strains increases the risk of developing Glässer's disease, while the non-virulent strains can provide some protection against the disease (Brockmeier et al., 2013). Although there are some suggestions in the literature of the existence of *M. hyorhinis* strains with different degree of virulence (J. H. Lin et al., 2006), and our data also support it, this has not been demonstrated. We detected specific ASVs of the pathogens that were found only in the disease farms and were not shared by the farms with different disease. The role of these specific *M. hyorhinis* ASVs in disease was supported by their detection in the clinical samples from those farms. Due to the limitations of the 16S rRNA gene amplicon sequencing, more studies with whole genome sequencing are needed. This result also reinforces the importance of study the microbiota at strain level, to better understand the intrinsic characteristics of the different strains and their role in the predisposition to the systemic infection.

In summary, different changes in the nasal microbiota composition were observed in weaning piglets from farms with polyserositis caused by either *M. hyorhinis* or *G. parasuis*. We

hypothesize that these changes might facilitate dissemination and the subsequent development of the systemic infections by *M. hyorhinis* and *G. parasuis*. The strain level resolution of the microbiota should be studied for virulent-strain detection, especially in the case of *M. hyorhinis* where there is a lack of studies addressing this issue.

MATERIAL AND METHODS

Farm Selection and sampling

Farms were selected based on the presence/absence of post-weaning polyserositis cases to study the nasal microbiota. Three groups of farms were included in the study, (Table 1). The first group, MH, was formed by two farms where polyserositis cases in nursery pigs were ascribed to *M. hyorhinis*. For such a purpose, samples from polyarthritis and/or polyserositis lesions were taken from animals at necropsy and *M. hyorhinis* was detected by qPCR (Clavijo et al., 2014) and/or isolation. In addition, four of the positive clinical samples (2 from RM and 2 from GE farms) were submitted at Servei de Genòmica, Universitat Autònoma de Barcelona to sequence the 16S rRNA gene using Sanger technology. All the samples from necropsies from MH farms were negative to isolation and/or PCR of *G. parasuis* (Olvera et al., 2012) and *S. suis* (Ishida et al., 2014). In each MH farm, nasal swabs were taken from ten 3–4 week-old piglets selected from different litters (two piglets per sow) in order to avoid a sow effect bias. The second group was formed by three control farms without polyserositis or respiratory cases in the last two years previous to the study (Group HC). The third group of farms, group GD, was composed of four farms in which the polyserositis problems were attributed to *G. parasuis*. Raw sequencing data from groups HC and GD belonged to a previous study with SRA Accession number SRP068182 (Correa-Fiz et al., 2016).

Sampling of piglets was done under institutional authorization and followed good veterinary practices. According to European (Directive 2010/63/EU) and Spanish (Real Decreto 53/2013) normative, this procedure did not require specific approval by an Ethical Committee (Chapter I, Article 1, 5 (f) of 2010/63/EU).

DNA Extraction and 16S rRNA Gene Amplicon Sequencing

Nasal swabs were placed in 500 µL de PBS, vortex for 30 s and stored at −80 °C until used for DNA extraction. DNA was extracted from 200 µL of the initial 500 µL PBS where the swabs were resuspended and eluted in 100 µL of PBS using the Nucleospin Blood (Macherey Nagel, Bethlehem, PA, USA) kit. Quantity and quality assessment of the DNA was performed using BioDrop DUO (BioDrop Ltd., Cambridge, UK). Samples were submitted for 16S rRNA gene amplicon sequencing using the Illumina paired-end 2 × 250 bp kit (MS-102-2003 MiSeq® Reagent Kit v2, 500 cycle) following the manufacturer's instructions. The library preparation for sequencing was performed within 24 h after the DNA extraction at Servei de Genòmica, Universitat Autònoma de Barcelona. The region amplified was V3–V4 that covers two hypervariable regions of the conserved gene, and it was sequenced according to the Illumina protocol (Klindworth et al., 2013a) as previously described (Correa-Fiz et al., 2016). The entire sequence dataset is available at the NCBI database, SRA accession number PRJNA717778.

Microbiota *in-silico* Analysis

The *in-silico* analysis was done using the plugin-based software Quantitative Insights into Microbial Ecology (Qiime) vs 2020.11 (Bolyen et al., 2019). First, after importing the raw sequencing data into Qiime2 (*q2-import*), we performed a quality check step and decided to trim the reads to a length of 240 bp based on the quality drop at the end of the reads. Denoising, trimming and quality-based filtering was performed with the *q2-dada2* plugin (Callahan et al., 2016), which also includes a chimera detection and consequent removal. Moreover, to improve the quality of our dataset, unassigned taxa was filtered out after aligning the ASVs against the Greengenes reference database (vs. 13.8) (McDonald et al., 2012), clustered at 88% identity with 65% of identity and over the 50% of query (Díaz et al., 2021).

With the rarefied curves of the richness, we extracted the proper sample-depth as a key parameter for the following diversity calculations. Diversity within each sample (alpha diversity) was estimated with

Shannon's entropy index, Observed Features and Chao index using *q2-core-metrics* plugin (Eren et al., 2012; C. Shannon & Weaver, n.d.). To compare the microbiota composition among the study groups (beta diversity), we calculated Jaccard, Bray Curtis and Unifrac (Jaccard, 1908; Lozupone & Knight, 2005; Sørensen, 1948) (weighted and unweighted) metrics and represented in the spatial coordinates with the Principal Components Analysis (PCoA). PERMANOVA (Z.-Z. Tang et al., 2016) tests were performed to analyze beta diversity clusters among study groups. Also, the Adonis function (Anderson, 2001) from Vegan package (Lab 2019) was used across the matrix distances in order to determine the percentage of explanation from the grouping variables analyzed.

Taxonomic assignment of the ASVs was performed by a pre-trained Naive Bayes classifier within the *q2-feature-classifier classify-sklearn* plugin (Bokulich et al., 2018) using the Greengenes database (Vs. 13.8) (McDonald et al., 2012). Classification of ASVs from *Glaesserella* and *Mycoplasma* genera was confirmed to species level using Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990) algorithm and NCBI RefSeq database (O'Leary et al., 2016). To find differentially abundant features across groups, analysis of compositional microbiomes (ANCOM) (Mandal et al., 2015) was performed. To process and analyze the data from the taxa assignment of the ASVs and the counts of the features we used *qiime2r* (Bisanz, 2021), *phyloseq* (McMurdie & Holmes, 2013), *tidyverse* (Wickham et al., 2019) R packages. Heatmap, PCoA and Venn diagrams plots were built in Rstudio (Rstudio Team, 2020) using *ggplot2* (Wickham, 2009), *ComplexHeatmap* (Gu, 2021) and *ggVennDiagram* (Gao, 2021) packages. The phylogenetic tree of the ASVs from *M. hyorhinis* and *G. parasuis* were built with Maximum likelihood algorithm using Qiime2 (Bolyen et al., 2019).

SUPPLEMENTARY MATERIAL

Supplementary Figures and Tables are available at Zenodo repository in this link: https://zenodo.org/record/7892375#.ZFQumOxBz66

STUDY II

Ceftiofur treatment of sows results in long-term alterations in the nasal microbiota of the offspring that can be ameliorated by inoculation of nasal colonizers

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ABSTRACT

Background

The nasal microbiota of the piglet is a reservoir for opportunistic pathogens that can cause polyserositis, such as *Glaesserella parasuis*, *Mycoplasma hyorhinis* or *Streptococcus suis*. Antibiotic treatment is a strategy to control these diseases, but it has a detrimental effect on the microbiota. We followed the piglets of 60 sows from birth to 8 weeks of age, to study the effect of ceftiofur on the nasal colonization by these pathogens and the nasal microbiota when the treatment was administered to sows or their litters. We also aimed to revert the effect of the antibiotic on the nasal microbiota by the inoculation at birth of nasal colonizers selected from healthy piglets. Nasal swabs were collected at birth, and at 7, 15, 21 and 49 days of age, and were used for pathogen detection by PCR and bacterial culture, 16S rRNA amplicon sequencing and whole shotgun metagenomics. Weights, clinical signs and production parameters were also recorded during the study.

Results

The composition of the nasal microbiota of piglets changed over time, with a clear increment of *Clostridiales* at the end of nursery. The administration of ceftiofur induced an unexpected temporary increase in alpha diversity at day 7 mainly due to colonization by environmental taxa. Ceftiofur had a longer impact on the nasal microbiota of piglets when administered to their sows before farrowing than directly to them. This effect was partially reverted by the inoculation of nasal colonizers to newborn piglets and was accompanied by a reduction in the number of animals showing clinical signs (mainly lameness). Both interventions altered the colonization pattern of different strains of the above pathogens. In addition, the prevalence of resistance genes increased over time in all the groups but was significantly higher at weaning when the antibiotic was administered to the sows. Also, ceftiofur treatment

induced the selection of more beta-lactams resistance genes when it was administered directly to the piglets.

Conclusions

This study shed light on the effect of the ceftiofur treatment on the piglet's nasal microbiota over time and demonstrated for the first time the possibility of modifying the piglets' nasal microbiota by inoculating natural colonizers of the upper respiratory tract.

INTRODUCTION

The relationship between the bacterial communities constituting the pig's microbiota from different tissues and the host has been an issue exponentially assessed during the last decade (Bergamaschi et al., 2020; Gresse et al., 2017; Pirolo et al., 2021). As it is observed in other studies and animal species, bacterial communities are niche-specific (Mach et al., 2021). A vast majority of the studies on the pig microbiome are focused on the gut, but in recent years more studies are investigating some other niches such as the skin, oropharyngeal, and nasal cavity (Fredriksen, Guan, et al., 2022; Pirolo et al., 2021; Strube et al., 2018).

Recent microbiota studies focused on the specific factors that contribute to microbiota shifts, such as breed, feed system, environment or antimicrobial treatment among others (Bergamaschi et al., 2020; Bosman et al., 2022; Obregon-Gutierrez et al., 2021). Usually, these factors have a deeper impact when the microbiota is unstable or immature and, therefore, more susceptible to external changes (Aranda-Díaz et al., 2022; Gibson et al., 2015; Rosier et al., 2018). Colonization of the respiratory tract starts at birth when most of the early colonizers are transferred from the dam and/or from environment (Obregon-Gutierrez et al., 2021). Several studies have shown the importance of the microbiota population structure in the development of posterior gut and respiratory diseases (Gresse et al., 2017; Mach et al., 2021; B. Wang et al., 2017). For that reason, cross-sectional studies are key to expand the knowledge about the dynamics of the host-microbiome interactions.

In swine industry, the prevention of diseases during the postweaning period has a fundamental impact on the subsequent production phases (J. Gao et al., 2019; Gresse et al., 2017). Weaning is a crucial moment in the piglet's life due to the implications in the maturation of the immune system and the intestine (X. Tang et al., 2022; L. Zheng et al., 2021). In addition, immune protection acquired from the mothers starts to decline during this stage and, together with the stress and changes associated with weaning (separation from the sows, mixing of litters and social challenge, nutritional changes…), can also trigger

some pathologies caused by bacteria present in the normal microbiota (pathobionts) (Costa-Hurtado et al., 2020; Ober et al., 2017). For instance, polyserositis is frequently observed in nursery pigs when members of the nasal microbiota, such as *Glaesserella parasuis*, *Streptococcus suis*, or *Mycoplasma hyorhinis*, spread systemically (Costa-Hurtado et al., 2020; Salogni et al., 2020).

Due to the lack of effective vaccines, the main strategy to control these diseases in piglets continues to be the use of antimicrobials (Costa-Hurtado et al., 2020; Hattab et al., 2022b). The use of antibiotics in sows is thought to reduce the transfer of pathogens to their offspring and control the pathogen load globally in the farm (VanderWaal et al., 2018). However, antimicrobials will also affect the composition of the beneficial microbiota (Correa-Fiz et al., 2019). Moreover, the indiscriminate use of antibiotics to control bacterial diseases (mostly in a metaphylactic approach) has been questioned due to the emergence of antimicrobial resistant bacteria. One of the alternative strategies to promote the health of piglets, and therefore reduce antibiotic usage in swine production, could be the use of microorganisms intended to provide benefits to the host (probiotics), and therefore pathogen exclusion. Interventions with probiotics may reduce or delay the colonization of the niche by pathogens. Probiotics are frequently used in humans but less commonly in pigs, where all of them target the gut microbiota (Hansen et al., 2021; Shin et al., 2019). However, the use of probiotics in the respiratory tract has only been investigated in a few studies where they proved their variable action against respiratory-associated infections (Al-Romaih et al., 2023; Luan et al., 2019; Mårtensson et al., 2022; Niittynen et al., 2012).

Here, we present the results obtained in a longitudinal study in a pig commercial farm with respiratory problems, where ceftiofur was applied to pregnant sows or piglets. The findings of this study revealed a more prolonged effect on the piglets' nasal microbiota when parenteral administration of ceftiofur was done to the sows than directly to newborn piglets. We also demonstrated for the first time the ability to modify the nasal microbiota of the piglets inoculating nasal colonizers at birth.

MATERIAL AND METHODS

Study design

Animal experimentation was performed following proper veterinary practices, under European (Directive 2010/63/EU) and Spanish (Real Decreto 53/2013) regulation and with the approval of the Ethics Commission in Animal Experimentation of the Generalitat de Catalunya (Protocol number 9211).

A field study was conducted in a 2-sites pig commercial farm with recurrent respiratory problems (site 1: gestation and lactation; site 2: nursery) located 15 kilometers apart. The farm had a 3-week lactation system with a total of 300 sows and a 3-week batch system and nursery facilities where animals remained until nine weeks of age. Four days before farrowing (D-4), 23 sows were intramuscularly treated (treated sows) with 10 mg of crystalline ceftiofur, a third-generation cephalosporin (Naxcel), while 31 sows remained untreated (non-treated sows). Sows were distributed in five different rooms in the lactation facilities (one group per room). Piglets were divided according to the treatment received by the sows, where 284 piglets born to treated sows and 407 born to non-treated sows were included in the study. To assess the effect of the antibiotic on the nasal microbiota of piglets when the treatment was applied either directly to them or to their sows, 3 groups were established (Table 1). At birth (D0), 129 piglets born to treated sows remained nontreated (TS group), while 115 piglets born to non-treated sows were intramuscularly treated with 1 mg of crystalline ceftiofur (Treated Piglet group = TP). As control, 118 piglets born to non-treated sows remained non-treated (control group).

On the other hand, to study the effect of early colonization with selected colonizers on the piglets' nasal microbiota, piglets were inoculated at D0 with a cocktail of 5 selected bacterial strains at 10^4 - 10^5 CFU/mL (dose of 200 µL) using a nasal spray (Table 1). These strains belonged to five different genera: *Vagococcus*, *Streptococcus*, *Moraxella*, *Rothia* and *Glaesserella*. The inoculated pigs were non-treated and originated from two different groups: 154 piglets were born

to ceftiofur-treated sows (Treated sow - Inoculated piglet = TS-IP) and 175 were born to non-treated sows (IP group).

Table 1. Number of sows and piglets per study group according to the sow and/or piglet treatment.

Sow treament (at 1 week pre-farrowing)	**Piglet treatment (at birth)**	**Group***
Non-treated (NTS)	Non-Treated (n=118 piglets from 10 sows)	Control
	Treated (n=115 piglets from 9 sows)	TP
	Inoculated (n=175 piglets from 12)	IP
Treated (TS)	Non-Treated (n=129 piglets from 11 sows)	TS
	Inoculated (n=154 piglets from 12 sows)	TS-IP

*For clarity, no acronym is explicitly mentioned when no treatment was applied.

The number of born and weaned piglets per litter and group was registered. All piglets (n=691) were followed during the first three weeks of age (lactation facilities) and after weaning a subset of them (n=490) was followed until 8 weeks of age (nursery facilities), collecting data on the general conditions and health status of the animals (Table 2). During the nursery period, all the animals that showed any clinical signs were treated with antibiotics (1.1mg/Kg gentamicin-amoxicillin) and were removed from the study. Body weight was recorded at birth (D0) and at weaning (D21) and average daily weight gain (ADWG) was calculated through this period and compared among groups using a mixed effect linear model using lme4 R package (Bates et al., 2015). The number of animals showing lameness or other clinical signs compatible with systemic infection and the associated mortality among groups were compared using Fisher test with Bonferroni correction (Rey & Neuhäuser, 2011). The number of born and weaned piglets per group was analyzed by a Chi-squared test.

Table 2. Study design and actions performed to followed piglets born to treated or non-treated sows.

Production phase	Day (D)	Action performed	Analysis	Non-treated sow			Treated-sow (TS)	
				Control (N=10)	TP (N=9)	IP (N=12)	TS (N=11)	TS-IP (N=12)
Lactation	D0	Piglet treatment		0	115	0	0	0
		Piglet inoculation		0	0	175	0	154
		Weight		118	115	175	129	154
		Nasal sampling	*M. hyorhinis, G. parasuis* and *S. suis* PCRs[a]	0	10	0	10	0
			Microbiota 16S sequencing	0	5	0	5	0
	D7	Nasal sampling	*G. parasuis* and *S. suis* culture[b]	10	9	12	11	12
			M. hyorhinis, *G. parasuis* and *S. suis* PCRs[c]	50	45	60	55	60
			Microbiota 16S sequencing	5	5	5	5	5
	D15	Nasal sampling	*G. parasuis* and *S. suis* culture[b]	10	9	12	11	12
			M. hyorhinis, *G. parasuis* and *S. suis* PCRs[c]	50	45	60	55	60
Nursery	D21	Weight		101	85	118	89	97
		Nasal sampling	*G. parasuis* and *S. suis* culture[b]	10	9	12	11	12
			M. hyorhinis, *G. parasuis* and *S. suis* PCRs[c]	50	45	60	55	60
			Microbiota 16S sequencing	5	5	5	5	5
			WGS metagenomics	5	5	5	5	5
	D49	Nasal sampling	*G. parasuis and S. suis culture*[b]	10	9	12	11	12
			M. hyorhinis, G. parasuis and S. suis PCRs[c]	25	20	25	25	25
			Microbiota 16S sequencing	5	5	5	5	5
			WGS metagenomics	5	5	5	5	5

[a]5 animals were randomly selected among the 10 samples for PCR testing.
[b]corresponded to one piglet per litter. [c]corresponded to five piglet per litter.

Sample collection and processing

Nasal swabs were taken from piglets belonging to each group at different timepoints (D0, D7, D15, D21, D49) for bacterial culture, PCR or microbiota analysis, as described in Table 2.

Nasal swabs were resuspended in 500 µL of PBS and kept refrigerated until arrival at research facilities where they were vortexed and stored at -20ºC. A total of 200 µL of the suspensions was processed using the Nucleospin Blood kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. Total extracted DNA was quantified using BioDrop DUO (BioDrop Ltd., Cambridge, UK) and stored at -20ºC for further processing.

PCR detection of *Glaesserella parasuis*, *Streptococcus suis,* and *Mycoplasma hyorhinis*

Swabs from 5 animals per litter from each group were used for pathogen detection by PCR. These five piglets were initially chosen randomly, and the same animals were tested by PCR at the different timepoints. If one of the piglets was not available at any timepoint, a littermate was chosen.

Detection of the pathogens present in the nasal cavity was done individually using specific PCRs on the 2µl from the total extracted DNA. For *G. parasuis,* a specific *vtaA* leader sequence PCR (Galofré-Milà et al., 2017b) was used for the detection and differentiation of virulent or non-virulent strains. Specific PCRs for *S. suis* detection were performed, as well as the specific PCRs for serotype 2 and serotype 9 as described before (Marois et al., 2004; Okura et al., 2014). In the case of *M. hyorhinis*, qPCRs were performed as previously described (Clavijo et al., 2014). Samples with a Ct value < 37 were considered positive.

PCR or qPCR results were expressed as percentage of positive samples per group and were compared through contingency tables with Chi-square test.

Genotyping of *Glaesserella parasuis* and *Streptococcus suis* isolates

Swabs from 1 animal per litter from each group were used for bacterial culture. As above described for PCRs, the same animals were used at the different timepoints.

Swabs were plated on chocolate agar and up to 4 colonies morphologically compatible with *G. parasuis* and 4 colonies compatible with *S. suis* were selected for identification and characterization.
In order to discriminate different strains, *G. parasuis* and *S. suis* isolates were genotyped by enterobacterial repetitive intergenic PCR (ERIC-PCR) with primers ERIC-1F (ATGTAAGCTCCTGGGGATTCAC) and ERIC-2R (AAGTAAGTGACTG GGGTGAGCG) (Versalovic et al., 1991). The PCR reaction mixture consisted of 3 mM of $MgCl_2$, 1.2 µM of each primer, 0.23 mM of dNTPs, 0.75 U of GoTaq® polymerase (Promega, Madison Wisconsin, USA) and 100 nanograms of DNA sample. Amplification was carried out with an initial denaturation of 94 °C for 2 minutes followed by 30 cycles of 30 seconds at 94 °C, 1 min at 50 °C and 2.5 min at 72 °C, and finally an extension of 20 min at 72 °C.

16S amplicon sequencing and *in silico* analysis

Five animals per group were selected for longitudinal analysis (D7, D15, D21 and D49) of the nasal microbiota by 16S sequencing. At farrowing (D0), 5 piglets from non-treated and 5 piglets from treated sows were selected.

The region targeted for the Illumina 16S amplicon sequencing was the V3-V4. This region was amplified using primers 341F (5′- 516 CCTAYGGGRBGCASCAG-3′) and 806R (5′-GGACTACNNGGGTATCTAAT-3′) (Klindworth et al., 2013b). Read length was 2x250bp and Illumina MiSeq technology was used (Klindworth et al., 2013b). The bioinformatic downstream analysis was done using Quantitative insights into microbial ecology (Qiime2) software toolkit (Bolyen et al., 2019, p. 2). Denoising and quality-control step was done using *q2-dada2* plugin (Callahan et al., 2016). The following step was to remove all the reads with no match with at least an 80% of identity against Greengenes (v13.8) rRNA database (McDonald et al., 2012) and 50% of length. Diversity analyses were done using *core-*

metrics plugin with a rarefied sample depth of 12,124. One sample was removed from the analysis due to not reach the query sample depth threshold. Alpha diversity was done using the Shannon diversity index (C. Shannon & Weaver, n.d.), and Chao index (Eren et al., 2012). Beta diversity distance matrices were calculated based on the weighted Unifrac index (Lozupone & Knight, 2005). A permutation-based analysis of variance (PERMANOVA) (Z.-Z. Tang et al., 2016) was done to test if the centroid of two or more groups were significantly different. The percentage of the variance explained by the study groups was calculated through the Adonis function from the Vegan package (lab, 2019) in R. Taxonomic assignment of each amplicon sequence variant was done using the Qiime2 classifier (*q2-feature-classifier* plugin) trained with the V3-V4 region from 16S gene and the Greengenes database (v13.8) (McDonald et al., 2012). Analysis of composition of microbiomes with bias correction (ANCOM-BC) (H. Lin & Peddada, 2020) function was done at each timepoint among all the groups to perform differentially abundant analysis at both amplicon sequence variant (ASV) level and collapsed at different taxonomic levels. All data was processed for tables, plots and figures using Rstudio (Rstudio Team, n.d.) and qiime2R (Bisanz, 2018/2021, p. 2), ggplot2 (Wickham, 2009) and tidyverse (Wickham et al., 2019) packages.

WGS metagenomic sequencing and *in silico* analysis

Extracted DNAs from the same swabs used for 16S sequencing at weaning (D21) and at the end of nursery (D49) were used for whole genome shotgun (WGS) metagenomics.

WGS metagenomic sequencing of the samples was done using Illumina Novaseq 6000 (2x150 bp) technology. The throughput required per sample was at least 15 Gigabases to acquire enough sequencing depth. Genomic data were analyzed under the biobakery3 (Beghini et al., 2021) metagenomic profiling workflow. Raw reads were QC processed and trimmed using Kneaddata pipeline. (Beghini et al., 2021), which uses Trimmomatic (Bolger et al., 2014) and Bowtie2 (Langmead & Salzberg, 2012). In addition, trimmed reads were aligned to the *Sus scrofa* reference genome v11.1 (Warr et al., 2020), to remove any read matching the host. Taxonomic profiling of each sample was assessed using

Metaphlan4 (Beghini et al., 2021; Blanco-Míguez et al., 2023) software, on cleaned read level. Functional profiling of all the samples was done through HUMAnN (Beghini et al., 2021) which quantifies gene families, EC enzyme modules, and pathways, using the UniRef (Suzek et al., 2007) and MetaCyc databases (Caspi et al., 2014). Differences among groups were estimated through a multivariate association analysis with linear models using MaAsLin2 R package (*Multivariable Association Discovery in Population-Scale Meta-Omics Studies | PLOS Computational Biology*, n.d.), embedded in the Biobakery3 toolkit. Abundances were passed through a basic filter requiring each pathway and taxa to have at least 0.01 % abundance in at least 3 % of all samples. Assembly of the clean reads was done using MetaSpades (Nurk et al., 2017). Taxonomic profiling of the metagenome-assembled genomes (MAGs) was done using Kraken2 (Wood et al., 2019). Analysis of the antimicrobial resistance genes (AMR) was done using Abricate software (Seemann, 2014) over the MAGS with the NCBI AMRFinderPlus database (Feldgarden et al., 2019).

RESULTS

Production parameters and health status

The mean number of liveborn piglets per litter was not significantly different among the groups (Table 3). No stillborns or mummies were recorded during farrowing. At weaning, inoculated piglets (TS-IP and IP) showed numerically higher values than the rest of the groups (Table 3).

Table 3. Number of liveborn and weaned piglets per litter, body weight and average daily weight gain per each treatment group.

	Farrow			Weaning			Farrowing to Weaning	
	Liveborn piglets		Body weight (Kg)	Weaned piglets		Body weight (Kg)	ADWG[#] (gr)	
Groups	Total	Mean per litter ±SD	Mean ±SD	Total	Mean per litter ±SD	Mean ±SD	n*	Mean ±SD
Control	118	13.10 ±2.76	1.71 ±0.35	108	10.80 ±1.46	5.59 ±1.08	101	0.18 ±0.05
TS	129	13.08 ±2.70	1.51 ±0.35	122	11.08 ±1.38	5.10 ±0.95	89	0.17 ±0.04
TP	115	12.58 ±2.68	1.38 ±0.34	97	10.77 ±1.25	5.60 ±1.34	85	0.19 ±0.06
IP	175	13.39 ±2.64	1.53 ±0.36	149	12.41 ±1.30	5.60 ±1.35	118	0.17 ±0.05
TS-IP	154	13.25 ±2.71	1.58 ±0.30	143	11.91 ±1.67	5.51 ±0.97	97	0.18 ±0.04

* The number of the animals weighed at weaning was lower than the number of weaned animals due to the fact that the rooms in the nursery facilities were smaller and not all the weaned animals included in the study could be allocated in them.[#]Only animals present at both timepoints were included

From the individual weights obtained at farrowing and at weaning (D21), the ADWG of the piglets was calculated and computed in a linear mixed effect model where the individual variation was computed as a random effect. No statistically significant differences between the mean body weight between groups at D0 (birth) and at D21 (weaning) were detected (Table 3).

During the lactation period (first three weeks of age), sporadic episodes of diarrhea were observed and, as a consequence, three animals from the control group were treated (gentamicin-amoxicillin). Through the nursery period, the main clinical signs observed were lameness and diarrhea, and piglets showing lameness were treated with gentamicin-amoxicillin (Table 4). To avoid any bias due to the effect of this antibiotic treatment on the microbiota, the gentamicin-amoxicillin treated animals were excluded from the study. During nursery, no significant differences were found in mortality rates among the groups. Notably, the TS-IP group showed a lower prevalence of lameness than the control group ($P < 0.05$, Chi-squared test; Table 4).

Table 4. Proportion (and percentages) of animals showing lameness and mortality rate compatible with respiratory or systemic disease at nurseries.
NA, not available.

Group	Lameness (affected/total)	Mortality rate	
		dead/total	dead/affected
Control	48/101 (41%)	8/ 101 (7.90%)	8/ 48 (16.6%)
TS	36/100 (28%)	9/100 (9%)	9/36 (25%)
TP	NA	NA	NA
IP	47/118(28%)	11/118 (9.32%)	11/47 (23.4%)
TS-IP	24/96 (15%)*	1/96 (1.04%)	1/24 (4.16%)

*Significantly different when compared to control group ($P < 0.05$).

Sow treatment induced long term changes in the nasal microbiota of the offspring

Globally, a total of 14,655,905 sequences were obtained by 16S amplicon (V3-V4) sequencing, with 12,084 different ASVs. Diversity analysis was calculated at a 12,124 read depth per sample, which required the elimination of one sample from the dataset due to low sequencing throughput (6,832 reads in a sample from the IP group at D7).

The effect induced by ceftiofur treatment in piglets' nasal microbiota was evaluated when administered to sows before farrowing. Alpha diversity was estimated longitudinally (Chao and Shannon indexes), from birth to fattening at four time points. Initially (D0), alpha diversity was not different in piglets born to treated or non-treated sows, but piglets born to treated sows showed a higher inter-individual variability (Fig. 1). The treatment on sows induced a temporal increase in the alpha diversity of the nasal microbiota of piglets at D7 (Shannon diversity index P=0.028) compared with the control group (Fig. 1). This rise in alpha diversity was not maintained through time and no significant differences were observed between TS and the control group at D21 or D49. When the beta diversity was analyzed, significant differences were detected at all sampling times, by either quantitative (Bray Curtis, weighted Unifrac) or qualitative (Jaccard, unweighted Unifrac) indexes. A stronger divergence was observed at D7 (Fig. 2), since the percentage

of explanation measured on weighted Unifrac between groups was higher (R^2 = 35%) at this timepoint compared to D21 (R^2 = 28%) and D49 (R^2 = 22%)

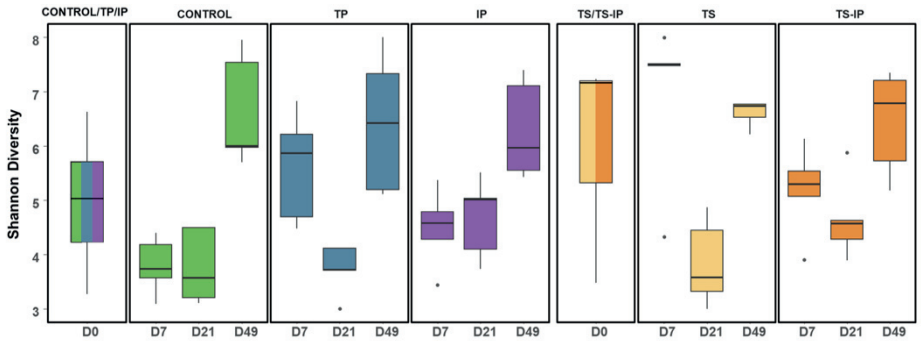

Figure 1. Alpha diversity (Shannon index) of nasal microbiota from piglet at different ages (D=days of age) and groups. Pregnant sows were treated with ceftiofur or remained untreated. A group of piglets born to treated sows remained nontreated (TS group), while piglets born to non-treated sows were treated with ceftiofur (TP group). A group of piglets were inoculated with selected colonizers of the upper respiratory tract either born to ceftiofur-treated sows (TS-IP) and to non-treated sows (IP group). As control, piglets born to non-treated sows remained non-treated.

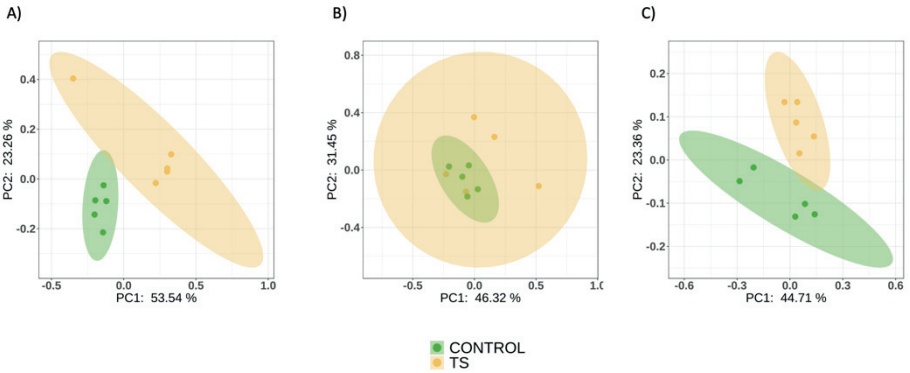

Figure 2. Beta diversity on Weighted Unifrac distance matrices. PCoA was performed at D7 (A), D21 (B) and D49 (C). Orange dots represent samples from piglets born to ceftiofur treated sows (TS group) and green dots represent non-treated piglets born to non-treated sows (Control).

Ceftiofur treatment directly administered to piglets in early life (TP) produced similar trends in alpha diversity than the treatment applied to their sows (TS). A significant increase in alpha diversity (P=0.009, Shannon diversity index) was detected at D7 in TP in comparison to the control group (Fig. 1). As observed in TP group, this increment was not maintained through time, and it was not observed at weaning (D21) or at

the end of the study (D49). When beta diversity of TP and control groups was estimated, we detected differences at D7 and D21 using quantitative (Fig. 3) and qualitative metrics. At D49, the difference between these two groups was only detected using Jaccard distance (qualitative measurement). Importantly, the effect was more evident at D7 (Adonis function, R^2 = 0.45, P = 0.012) than at the other timepoints (Adonis function, R^2= 0.24, and R^2= 0.15, at D21 and D49 respectively), underlying the different microbial composition in TP compared to the control soon after the antibiotic treatment.

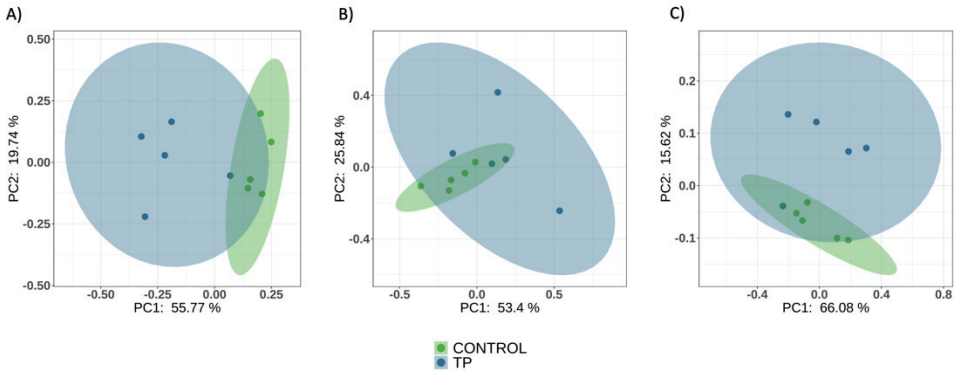

Figure 3. Beta diversity on Weighted Unifrac distance matrices. PCoA was performed at D7 (A), D21 (B) and D49 (C). Blue dots represent samples from ceftiofur treated piglets born to non-treated sows (TP group) and green dots represent non-treated piglets born to non-treated sows (Control).

Lastly, both treated groups (TP and TS) were compared to determine if the changes led by the antibiotic treatment were similar when administered to the piglets or to their sows. No significant differences were observed in alpha diversity at any sampling point. When beta diversity was analyzed, Jaccard, unweighted Unifrac and Bray Curtis metrics detected significative differences at D7 (P=0.01, P=0.026 and P=0.03, respectively), but this difference was not observed using weighted Unifrac (Additional file 1: Fig. S1). Quantitative differences (weighted Unifrac and Bray Curtis) were not maintained at D21, while they were still detected with the qualitative analysis (Jaccard and unweighted Unifrac). Similar results that those found at D7 were obtained at D49 (unweighted Unifrac P = 0.006; Jaccard P = 0.008; Bray Curtis P = 0.012). Differences in the significance of quantitative and

qualitative metrics pointed out that these differences were provoked by bacterial communities with low relative abundance.

To understand the microbial changes leading to these differences, we performed an analysis of the compositional microbiome with bias correction (ANCOM-BC) at each timepoint based on either amplicon sequence variants or at different taxonomic levels. At D7, we found 488 ASV in TS and 205 ASVs in TP that were differentially abundant when compared to the control group (ASVs; Additional file 2: Table S1), which was in agreement with the higher alpha diversity observed in this group. A significant increase in the relative abundance of ASVs from *Bacteroidales* (3.972% in TS, 3.534% in TP versus 0.357% in control) and *Clostridiales* (18.487% in TS, 7.733% in TP versus 2.452% in control) was observed at D7 in both antibiotic-treated groups. On the other hand, typical members of the nasal microbiota were decreased in the treated groups with respect to the non-treated control group, including *Glaesserella*, *Moraxella* and *Rothia* (Table 5).

Table 5. Relative abundance of genera belonging to the core microbiota from control group at weaning from each group at D7 and D21.

	D7			D21		
Taxa	**Control**	**TS**	**TP**	**Control**	**TS**	**TP**
p__Proteobacteria;o__Pasteurellales;f__Pasteurellaceae;g__Glaesserella	0.0183	0.0601	0.0017	0.2754	0.1094	0.1490
p__Bacteroidetes;o__Flavobacteriales;f__[Weeksellaceae];g__Bergeyella	0.0000	0.0250	0.0000	0.1063	0.2005	0.0679
p__Proteobacteria;o__Pseudomonadales;f__Moraxellaceae;g__Enhydrobacter	0.1252	0.0013	0.0010	0.0876	0.1265	0.1616
p__Firmicutes;o__Lactobacillales;f__Streptococcaceae;g__Streptococcus	0.0243	0.0249	0.0145	0.0320	0.0746	0.0368
p__Proteobacteria;o__Caulobacterales;f__Caulobacteraceae;g__Caulobacter	0.1005	0.0773	0.1593	0.0761	0.0801	0.1341
p__Proteobacteria;o__Pseudomonadales;f__Moraxellaceae;g__Moraxella	0.3518	0.0500	0.0476	0.0673	0.0298	0.0190
p__Proteobacteria;o__Rhizobiales;f__Rhizobiaceae;g__Rhizobium	0.0495	0.0307	0.0705	0.0402	0.0718	0.0785
p__Firmicutes;o__Lactobacillales;f__Lactobacillaceae;g__Lactobacillus	0.0174	0.0530	0.1236	0.0022	0.0095	0.0052
p__Actinobacteria;o__Actinomycetales;f__Micrococcaceae;g__Rothia	0.0069	0.0232	0.0100	0.0173	0.0026	0.0058
p__Bacteroidetes;o__Bacteroidales;f__Bacteroidaceae;g__Bacteroides	0.0008	0.0087	0.0214	0.0025	0.0018	0.0044
p__Proteobacteria;o__Rhizobiales;__;__	0.0495	0.0307	0.0705	0.0003	0.0009	0.0017
p__Bacteroidetes;o__Bacteroidales;f__Prevotellaceae;g__Prevotella	0.0008	0.0090	0.0064	0.0012	0.0015	0.0010
p__Firmicutes;o__Clostridiales;f__Ruminococcaceae;g__	0.0008	0.0046	0.0010	0.0020	0.0019	0.0009
p__Proteobacteria;o__Pseudomonadales;f__Moraxellaceae;g__Acinetobacter	0.0151	0.0996	0.0816	0.0018	0.0048	0.0151
p__Proteobacteria;o__Rhizobiales;f__Bradyrhizobiaceae;g__Bradyrhizobium	0.0064	0.0029	0.0078	0.0034	0.0035	0.0113
p__Proteobacteria;o__Enterobacteriales;f__Enterobacteriaceae;g__Escherichia	0.0018	0.0104	0.0229	0.0055	0.0058	0.0071
p__Firmicutes;o__Clostridiales;f__Clostridiaceae;g__Clostridium	0.0025	0.0247	0.0047	0.0009	0.0014	0.0007
p__Proteobacteria;o__Burkholderiales;f__Comamonadaceae;g__Variovorax	0.0060	0.0047	0.0083	0.0029	0.0072	0.0017
p__Firmicutes;o__Clostridiales;f__;g__	0.0025	0.0247	0.0047	0.0020	0.0019	0.0009
p__Firmicutes;o__Turicibacterales;f__Turicibacteraceae;g__Turicibacter	0.0009	0.0049	0.0016	0.0013	0.0002	0.0015

NA, not available.

* Significantly different when compared to control group ($P < 0.05$).

At D21, the number of differential ASVs was higher when comparing the control group with TS (92 ASVs) than with TP (68 ASVs). Among the ASVs affected by the antibiotic treatment, we found

an ASV from *Mycoplasma* genus that was decreased in TS and absent in TP. This relative decrease in *Mycoplasma* in TP group was assigned, at least partially, to *M. hyorhinis* by PCR (Additional file 3: Table S2). Differentially abundant ASVs among the three groups (TS, TP and control) belonged to nasal-associated taxa, such as *Streptococcus* (more relatively abundant in TP), *Moraxella* and *Glaesserella* (both more relatively abundant in the control group). Interestingly, the same evidence was not observed at the genus level, suggesting that the antibiotic treatment in sows or piglets selected specific strains in each case. Within *Glaesserella genus, G. parasuis* is the only known member of the swine microbiota, for which a virulence-specific PCRs is available. We observed different colonization dynamics by virulent or non-virulent strains in the three groups, especially at early time points (Additional file 3: Table S2). The differences in ASVs were also supported by the isolation of colonies with a different fingerprinting profile in ERIC-PCR in the different groups. At genus level, not significant differences were detected, suggesting that the distribution of the different ASVs compensate each other yielding similar relative abundances in the groups. At D49, a global increase in the number and abundance of ASV was evident in all groups (as indicated in alpha diversity) and corresponded mostly to ASVs from *Bacteroidales* (9.40% in TS, 13.01% in TP and 12.56% in control) and *Clostridiales* (23.79% in TS, 21.54% in TP and 40.63% in control). At the end of nursery 593 ASVs were differentially abundant among the three groups (Additional file 4: Table S3). At genus level, some differentially abundant genera showed to be group-specific, such as *Mycoplasma,* whose abundance was higher in the control group (1.28% versus 0.15% in TP and 0.33% in TS). In agreement, higher prevalence of *M. hyorhinis* was detected in the control group by PCR (95% versus 74% in TP and 66% in TS). Sixteen genera were differentially abundant comparing the three groups (TS, TP and control), including some nasal-associated commensals: *Actinobacillus* (higher in TP), *Bordetella* (higher in TS), *Lactobacillus* (higher in TP), *Staphylococcus* (higher in TS) and a genus from the *Pasteurellaceae* family (higher in TS) (Additional file 5: Table S4). Ceftiofur treatment did not affect the prevalence of *S. suis* as detected by PCR, with a prevalence of over 90% at D7 and 100% at weaning and onwards in the three groups.

Inoculation of early colonizers of the upper respiratory tract modifies the nasal microbiota of the piglets.

Inoculation of non-treated piglets with selected colonizers (IP group) had an impact on their nasal microbiota. At D7, the mean alpha diversity measured by Shannon diversity index was higher in the inoculated IP piglets (Fig. 1) compared to the control group, although not significant at any timepoint (Kruskal-Wallis, P=0.075, 0.14 and 0.25 for D7, D21 and D49, respectively). The microbiota composition estimated through beta diversity based on weighted Unifrac distance matrix was calculated for each timepoint (Fig. 4). Control and inoculated groups showed statistically different beta diversity at each timepoint (PERMANOVA, P=0.02, P=0.01 and P=0.043 for D7, D21 and D49, respectively). The percentage of explanation attributed to the inoculation of the piglets (estimated through the Adonis function on the weighted Unifrac distance matrix) was 53% at D7, 44% at D21 and 36% at D49. Differential abundance analysis performed with ANCOM-BC methodology showed a total of 379 differential ASV, including four of the ASVs from the inoculated bacteria *Moraxella*, *Rothia*, *Streptococcus* and *Glaesserella*, which showed higher abundance in the IP group compared with the control group at D7 (Fig. 5), indicating that four out of five strains inoculated were able to colonize the nasal cavity of the piglets. The ASVs corresponding to the inoculated *Glaesserella*, *Streptococcus* and *Moraxella* were also present at weaning (D21), although significative differences were only detected with the *Streptococcus* ASV. These ASVs were not significantly different at the end of nursery where only *Glaesserella* and *Streptococcus* were detected at this latter timepoint. The inoculated *Glaesserella* strain was also recovered by culture at all the timepoints.

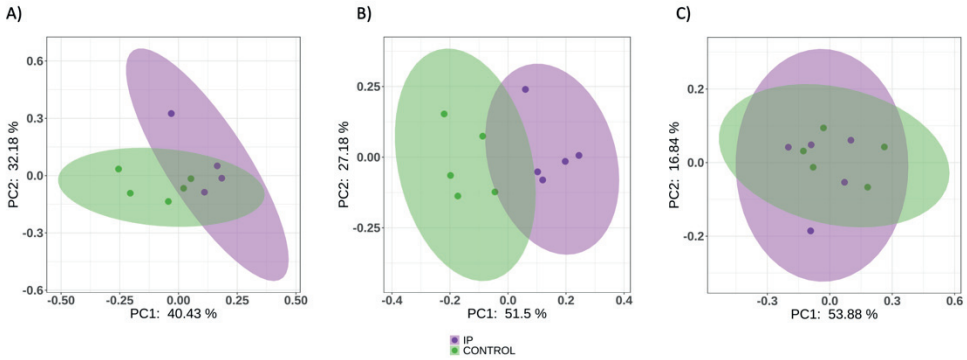

Figure 4. Beta diversity on Weighted Unifrac distance matrices. PCoA was performed at D7 (A), D21 (B) and D49 (C). Purple dots represent samples from inoculated piglets with selected colonizers born to non-treated sows (IP group) and green dots represent non-treated piglets born to non-treated sows (Control).

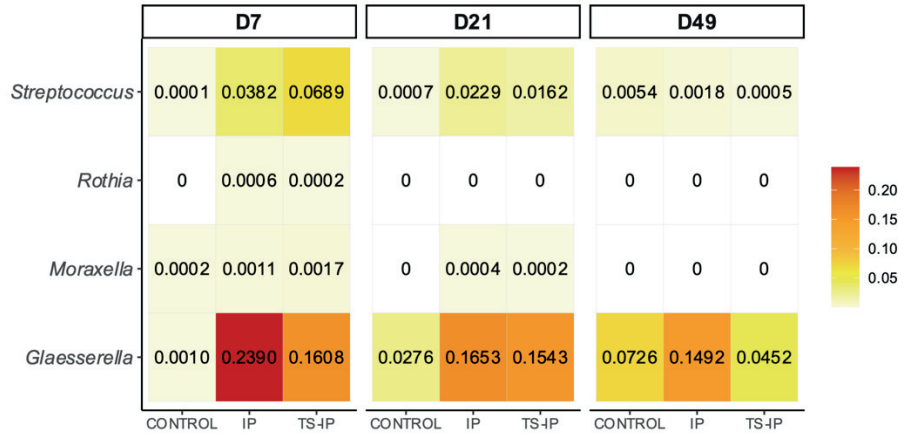

Figure 5. Heatmap representing the relative abundance (percentage) of selected colonizers inoculated at birth in the inoculated (IP and TS-IP) and the control groups at D7, D21 and D49.

The colonization of the piglets by the selected colonizers seemed not to be favored by treating the sows with ceftiofur. When alpha diversity (measured through Shannon index) of inoculated piglets from treated or non-treated sows (IP vs TS-IP) was compared, no significant differences were detected at any of the time points analyzed (Kruskal Wallis, $P > 0.05$; Fig. 1). Differences in microbiota composition (beta diversity) were not detected with any of the metrics/distances analyzed (Jaccard, Bray Curtis and Unifrac; Fig. 6) at any of the sampling timepoints (IP vs TS-IP; Bray Curtis PERMANOVA, P=0.396). The nasal microbiota composition of the two inoculated groups was not

different at D7, although it was different for both groups when compared to the control (PERMANOVA, TS-IP vs control, *P*=0.008 and IP vs control, *P*=0.013, at D7). ANCOM-BC analysis showed a total of 379 ASVs at D7, including the ASV of the inoculated *Rothia* (0.0006% in IP and 0.0002% in TS-IP, while absent in control group). Similar findings were observed at D21 (PERMANOVA, IP vs TS-IP, *P*=0.142; IP vs control *P*=0.011; TS-IP vs control, *P*=0.007) in beta diversity analysis, but at this timepoint none of the colonizers were differentially abundant. At the end of nursery (D49), inoculated groups were not significantly different (IP vs TS-IP; Bray Curtis PERMANOVA, *P*=0.062). At this timepoint, only *Glaesserella* and *Streptococcus* were detected in the samples, but no significant differences were found in the differential abundance analysis (ANCOM-BC). Inoculation of the selected nasal colonizers reduced the divergence of the microbiota composition produced by the treatment of the sows. The differences detected at D7 and D21 in beta diversity were absent at the end of the study, indicating TS-IP piglets had a similar composition to the control (*P*=0.163, with all beta diversity metrics used, while piglets born to treated sows but not inoculated (TS) presented different composition than control at D49. The IP group also showed a different microbiota than control at D49 (*P*=0.008). Interestingly, the inoculated colonizers were found in higher relative abundance in IP than in TS-IP.

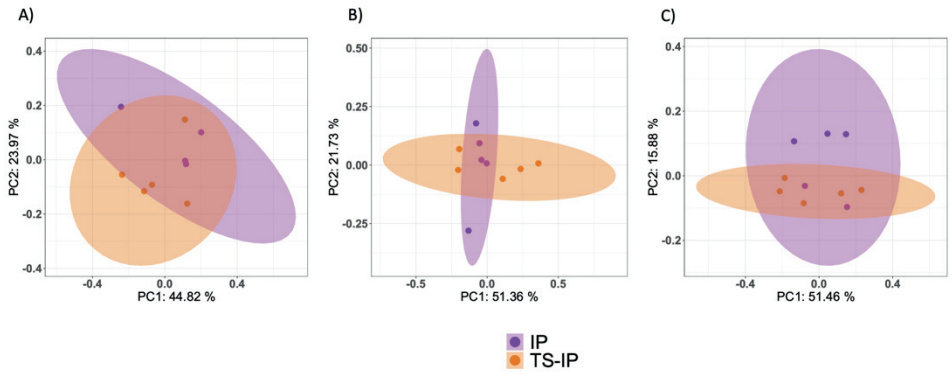

Figure 6. Beta diversity on Weighted Unifrac distance matrices. PCoA was performed at D7 (A), D21 (B) and D49 (C). Purple dots represent samples from inoculated piglets with selected colonizers born to non-treated sows (IP group) and dark orange dots represent inoculated piglets born to treated sows (TS-IP).

WGS metagenomics confirmed the differences observed in beta diversity between weaning and the end of nursery in all groups.

A total of 143 species and 99 genera were identified in the nasal microbiota samples from D21 and D49. In the control group, we identified a total of 10 genera and 14 species at weaning (D21), and the composition of the nasal microbiota was clearly modified through the incorporation of other members at D49 (Fig. 7A Top 50 species z-score control group D21 y D49). The divergence between D21 and D49 was also observed in piglets from the rest of the groups in a clustering analysis by average abundance (Z-score) at different taxonomic levels, including species (Additional file 6: Fig. S2, Bray Curtis top 25 species D21, D49 in Ts, TP and control). At D21, the top 10 most abundant genera in all groups were *Glaesserella*, *Moraxella*, *Bergeyella*, *Mycoplasma*, *Streptococcus*, *Pasteurella*, *Neisseria*, *Mannheimia*, *Lactobacillus* and *Acinetobacter*, which belong to taxa commonly associated to nasal microbiota. At the end of the nursery (D49), a plethora of species from taxa classically associated to gut microbiota and absent at weaning was detected. The same colonizers were detected at weaning as described for the 16S sequencing data and expanded their detection at the end of nursery detecting not only the *Rothia* at the end of nursery but *Glaesserella* strain as well.

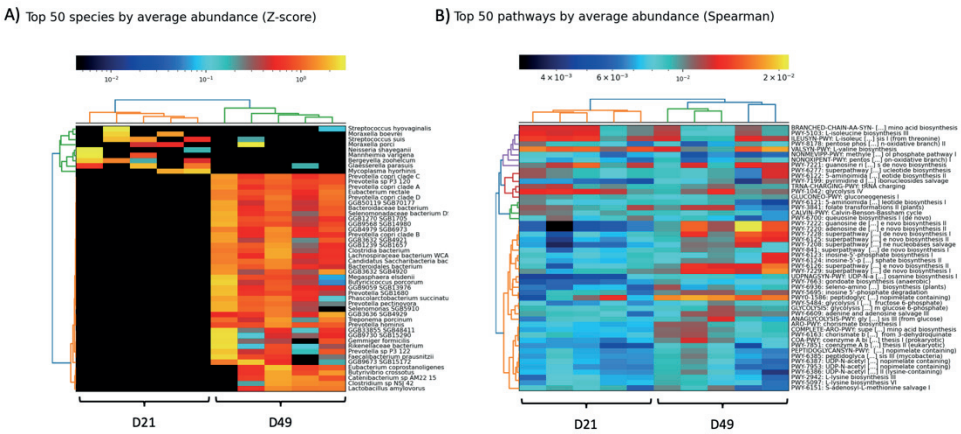

Figure 7. Heatmap representing the top 50 species by average abundance clustered with Z-score normalization in non-treated piglets born to non-treated sows at D21 and D49 (A). Top 50 pathways by average abundance by Spearman correlation coefficient in control group at D21 and D49 (B).

Several species within *Archaea* domain were affected by the antibiotic treatment. While they were not found in any group at weaning, at the end of the nursery they were found more relatively abundant in the control and the IP group than the piglets born to treated sows (TS and TS-IP), although in less than one percent in all groups. *Archaea* were absent in the TP group at this latter timepoint as well.

A total of 314 pathways and 1580 enzymes were found considering all samples. Most of the pathways were present in all groups (Additional file 7: Fig. S3), except TS group which showed several specific pathways at D21, although represented in very low relative abundance. The differences found at taxa level between weaning and the end of nursery were also found in the functional profiling but were not so remarkable (Fig 7B).

In the control group, 12 significantly different pathways were detected (through multivariable association analysis with linear model) when compared longitudinally (between D21 and D49 timepoints).These pathways were not among the top-50 most abundant pathways, and included several pathways in relative higher abundance at D21 (peptidoglycan maturation, heme b biosynthesis II, and two lipid IVA - precursor of lipid A- biosynthesis pathways) and others with higher abundance at D49 (anaerobic degradation of acetylene, degradation of stachyose, degradation of fucose and rhamnose, incomplete reductive TCA cycle, de novo biosynthesis of NAD from aspartate, L-arginine biosynthesis and two pathways of biosynthesis of pyrimidine).

Focusing on differences due to the antibiotic treatment at each time point, we detected few differential pathways, which were in higher abundance in the TS group (L-glutamate and L-glutamine biosynthesis at D21 but also at D49, superpathway of anaerobic sucrose degradation at D21, glycolysis-TCA-Glyox-bypass: superpathway of glycolysis at D49, and L-glutamine biosynthesis II at D21) when compared to TP and control groups.

In inoculated groups, we observed several pathways differentially increased at D21. Interestingly, these pathways increased in all groups at D49, indicating that these functions appeared earlier due to intervention, such as stachyose degradation, NAD *de novo* biosynthesis from aspartate, dTDP-beta-L-rhamnose biosynthesis (Additional file 8; Fig. S4). On the other hand, three pathways were

found at lower abundance in both inoculated groups at D21 when compared to the control group, showing a delay in their natural appearance: superpathway of branched chain aminoacid, L-isoleucine biosynthesis I (from threonine) and L-isoleucine biosynthesis III. In addition, some differences were specifically observed in each inoculated group. The IP group showed two differentially abundant pathways: one in higher abundance at D49 (chitin derivatives degradation), and a second with lower abundance also at D49 (beta-(1,4)-mannan degradation). In TS-IP group, we observed five pathways that were more abundant at D21: incomplete reductive TCA cycle, NAD salvage pathway II (PNC IV cycle), 4-deoxy-L-threo-hex-4-enopyranuronate degradation, superpathway of arginine and polyamine biosynthesis, and purine nucleobases degradation II (anaerobic), where the latter three pathways appeared in lower relative abundance at D49 in the same group TS-IP (Additional file 8; Fig. S4).

The divergence observed in the taxonomic profiles among the groups was not so evident at the functional level, suggesting that different microbial communities may be responsible for similar functions.

A higher number of beta-lactamase genes were detected in antibiotic-treated piglets

The genes involved in antibiotic resistances (resistome) were predicted using the AMRFinderPlus database by comparing the MAGs present in each group at D21 and D49. For each group and timepoint, the total number of genes associated to AMR found in each group is listed in Additional file 9: Table S6. Globally, the number of unique resistance genes increased significantly over time in all groups except TS. This TS group showed the highest number of unique resistance genes at D21 (31 genes from 17 families), a number significantly higher than the control (Fisher test with Bonferroni correction, P=0.026), which presented the lowest number at D21 (14 genes from 9 families). The number of unique AMR genes after the inoculation of colonizers was not different from the control group at D21 or D49. The counts of genes from the predicted resistome belonging to different antibiotic families is represented in Fig. 8.

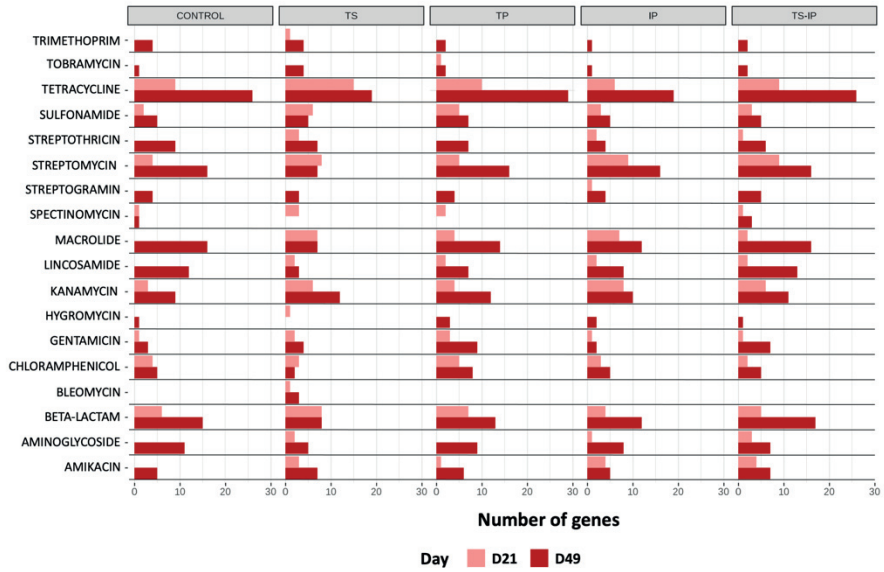

Figure 8. Number of genes associated with the different antibiotic classes found in the MAGs in the different groups and timepoints (D21 and D49). Pregnant sows were treated with ceftiofur or remained untreated. A group of piglets born to treated sows remained nontreated (TS group), while piglets born to non-treated sows were treated with ceftiofur (TP group). A group of piglets was inoculated with selected colonizers of the upper respiratory tract either born to ceftiofur-treated sows (TS-IP) and to non-treated sows (IP group). As control, piglets born to non-treated sows remained non-treated.

Since ceftiofur was the antibiotic administered in this study, we focused on the genes related to this resistance. In general, we detected 7 genes for class-A beta-lactamases: *cfxA* and *cfxA6* (broad spectrum), *blaTEM-122* (inhibitor-resistant broad spectrum), *blaROB-1* (cephalosporin hydrolyzing enzyme), *blaACI-1* and *blaCTX-M-32* (extended spectrum) and *blaBRO-1* (Fig. 9). The number of these genes increased with age in all groups. No major differences were detected among the distribution of these genes between the groups, except for *blaCTX-M-32* and *blaTEM-122*, which were found only in the TP group at D21 and D49, respectively.

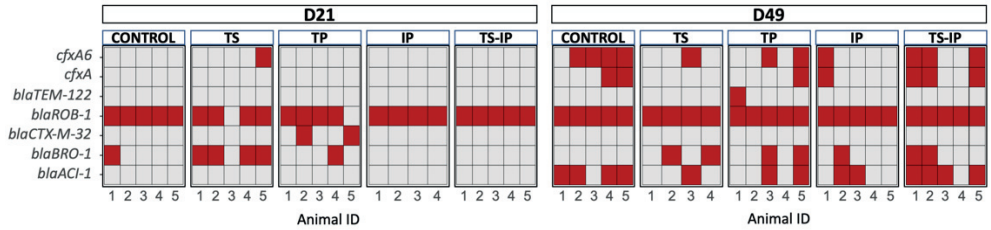

Figure 9: Beta-lactam genes presence (in red) individually in piglets by group and timepoint (D21 and D49). Pregnant sows were treated with ceftiofur or remained untreated. A group of piglets born to treated sows remained nontreated (TS group), while piglets born to non-treated sows were treated with ceftiofur (TP group). A group of piglets were inoculated with selected colonizers of the upper respiratory tract either born to ceftiofur-treated sows (TS-IP) and to non-treated sows (IP group). As control, piglets born to non-treated sows remained non-treated.

DISCUSSION

This study was motivated by two main questions, firstly to assess the impact of the ceftiofur treatment either in sows or piglets over time on their nasal microbiota and secondly, to explore the possibility of modifying it by inoculation at birth of natural colonizers selected from healthy piglets. The administration of ceftiofur had a longer impact on the nasal microbiota composition of piglets when administered to their sows before farrowing than directly applied to the piglets at birth. The effect of ceftiofur on nasal microbiota composition was partially reverted by the inoculation of nasal colonizers to newborn piglets and was accompanied by an improvement in piglet health. In addition, the prevalence of antibiotic resistance genes increased over time in all groups, being higher at weaning in the group of piglets born to treated sows. The selection of more beta-lactam resistance genes (*blaCTX-M3*and *blaACI-1*) was also observed when ceftiofur was administered directly to the piglets. Of note, our findings may be farm specific since one of the major factors involved in shaping the microbiota of the animals is the environment (Fredriksen, Guan, et al., 2022; Megahed et al., 2019; Slifierz et al., 2015). However, the strength of this study relies in the number of animal and the longitudinal approach to study the nasal microbiota, together with the two sequencing techniques used at a high sequencing depth. In agreement with Pirolo et al. (Pirolo et al., 2023), we found less diversity in the samples sequenced by WGS metagenomics than by amplicon sequencing, although the specific results are not

completely comparable due to the different study design (age of the animals, sampling technique, bioinformatic analysis, etc).

The nasal microbiota composition of the followed-up piglets switched from a clear predominance of *Firmicutes*, *Proteobacteria* and *Bacteroidetes* at weaning to a microbiota composition with an important contribution of *Clostridiales* and other taxa classically found in gastrointestinal tract (Slifierz et al., 2015). This can be explained by the change from milk to solid feed (Slifierz et al., 2015) and the natural rooting behavior of the piglets that put the nostrils in contact with fecal material (Mkwanazi et al., 2019). In the postweaning barn, the higher animal density worsens the air quality (increased levels of ammonia and dust), probably contributing to an inflammation state in the nose allowing the presence of anaerobic taxa. In fact, the increased presence of anaerobic bacteria in the human sinus correlates with inflammation and chronic diseases related to failure in immune system priming by nasopharyngeal microbiota (Kumpitsch et al., 2019). These changes underline the importance to conduct longitudinal studies to understand the evolution of the nasal microbiota over time.

As expected, ceftiofur treatment affected the microbiota composition. However, early after administration, the antibiotic induced an unforeseen increase in the diversity of the piglets' nasal microbiota. This finding confronts many studies that have shown that antimicrobial administration reduces bacterial diversity in different ecological niches (Correa-Fiz et al., 2019; Huang et al., 2022; Ramirez et al., 2020; Raymann et al., 2018). However, this was a transitory increment in the diversity derived from the presence of a plethora of bacterial taxa that are not consistently described in the respiratory microbiota of suids, whose presence can be considered as dysbiotic. These intrusive bacteria belonged mainly to taxa that could be traced back to the environment and were probably occupying the space available on the nasal mucosa after the drastic reduction of the nasal-associated bacteria, caused by the antibiotic treatment. In the long term, our data showed that the effect of the antimicrobial treatment lasted longer when administered to the sows compared to the direct administration to the piglets. This finding is in agreement with other studies where the sow contact with the newborn piglets was reported as one of the major drivers in the nasal microbiota composition (Obregon-Gutierrez et al., 2021). Management of the sow

seems to have an important impact on the piglet's nasal microbiota at least during the first weeks of age when the microbiota is still not stable (Slifierz et al., 2015), as previously observed when vaccination performed on sows modified the nasal microbiota of piglets (Blanco-Fuertes et al., 2022). Interestingly, inoculation of nasal colonizers reverted the effect of the ceftiofur administration in sows, reducing the fluctuations in diversity and modulating the microbiota towards a more stable scenario. Inoculation delayed the appearance of virulent strains of *G. parasuis* and we detected different dynamics of the prevalence of *G. parasuis*, *S. suis* and *M. hyorhinis* strains, but it is difficult to correlate these changes to the clinical status. However, the group of piglets born to treated sows that were inoculated with colonizers showed better clinical status after weaning, probably due to the reduction of the transmission of pathogens from the sows, which were replaced by natural members of the microbiota. In humans, the impact of antibiotic administration before delivery has been also associated with alteration in the infant's microbiota and health (Barnett et al., 2023; Prescott et al., 2021). Our findings suggest that the inoculation of potential beneficial colonizers may open a strategy to promote newborn health in case the mother needs to be treated. In fact, some studies have tested nasal probiotics against respiratory infections in broilers and humans with positive results (Al-Romaih et al., 2023; Luan et al., 2019).

In the present study, ceftiofur treatment, either of the sows or the piglets, did not improve the health status or the productivity of the piglets, indicating that metaphylactic treatments can be avoided without deleterious effects on production. In addition, we have observed that, with age, piglets showed an increasing number of antibiotic resistances, in agreement with previous reports (Gaire et al., 2023). In the current scenario of antimicrobial reduction in farms, the manipulation of the microbiota to maintain animal health appears as a promising strategy. Here, we have demonstrated that modification of the piglets' nasal microbiota by the inoculation of natural colonizers is possible. However, it would be essential to select natural colonizers free of antimicrobial resistances, especially those in mobile elements that could be easily transferable. Interestingly, the inoculated bacteria seemed to colonize better in piglets born to non-treated sows, probably due to some beneficial interactions with other members of the nasal microbiota.

CONCLUSIONS

This study shed light on the influence of the antimicrobial treatment on the piglets' nasal microbiota over time. We have demonstrated that ceftiofur treatment has a longer effect on the piglet's nasal microbiota when it is administered to the sow instead of directly to the piglet. Moreover, the effects of the sow antibiotic treatment on piglet nasal microbiota were partially reverted by inoculating a pool of nasal colonizers. This might represent a strategy to improve the pig's health by using a non-invasive alternative to antibiotics.

SUPPLEMENTARY MATERIAL

Supplementary Figures and Tables are available at Zenodo repository in this link:
https://zenodo.org/record/7892375#.ZFQumOxBz66

STUDY III

Sow vaccination against virulent *Glaesserella parasuis* shapes the nasal microbiota of their offspring

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ABSTRACT

Glaesserella parasuis is the etiological agent of Glässer's disease, a common pathology in the pork industry with higher prevalence in the postweaning period. Vaccination is one of the strategies to control this disease. Here, we investigated the effect that sow vaccination against virulent strains of *G. parasuis* had in the nasal microbiota of their offspring. Nasal swabs from fifteen days-old piglets from vaccinated (vs-P, n = 11) and unvaccinated sows (cs-P, n = 11) were obtained and DNA was extracted for 16S amplicon sequencing. Microbiota composition was different, with lower diversity in vs-P, and a strong clustering of the groups in beta diversity analysis. Among the 1509 sequences associated to either study group, all the sequences classified *G. parasuis* (10 ASVs) had lower relative abundance in the vs-P group. A list of 32 inferred metabolic pathways were statistically different between groups. A distinctive structure of the two microbial networks was detected, with modules in the cs-P not conserved in the vs-P network. In conclusion, vaccination of the sows had a large effect in the microbiota composition of their offspring that went beyond the effect on the targeted pathogen. The mechanisms underneath these changes may include alteration of the microbiota network due to the elimination of the targeted pathogen and/or immunological changes.

Keywords: *Glaesserella parasuis*, sow vaccination, virulent strains, nasal microbiota, 16S.

INTRODUCTION

Glaesserella parasuis is the etiological agent of Glässer's disease, an infectious disease with an important economic impact on swine production (Costa-Hurtado et al., 2020). This microorganism is an early colonizer of the upper respiratory tract and is part of the healthy nasal microbiota during all the stages of pig's life. In order to reduce the prevalence of Glässer's disease, different strategies have been used. Antimicrobial treatments and vaccines are the main tools to control disease (Zhao et al., 2017), although the global concern about rising antimicrobial resistances makes vaccination the method of choice. The natural source of this microorganism for piglets resides in the dams and colonization is known to occur very early in life (Cerdà-Cuéllar et al., 2010). Vaccination of the sows increases the specific antibodies transferred to their offspring through colostrum intake (Devillers et al., 2007) and may delay the colonization by *G. parasuis* (Cerdà-Cuéllar et al., 2010). Thus, sow vaccination can be a good strategy to decrease the prevalence of the virulent strains of this microorganism in the offspring, when using a vaccine that specifically targets these strains (López-Serrano et al., 2021).

The microbiota, known as the set of microorganisms, that inhabit a host (NIH HMP Working Group et al., 2009; Robinson & Pfeiffer, 2014), has different essential functions for the host such as nutrition, preservation of the mucosal homeostasis, prevention of pathogen colonization, and maturation of the immune system (Brestoff & Artis, 2013). Nasal microbiota, as gut microbiota, is affected by a wide range of factors in piglets, such as age, contact with the mother or environment (Obregon-Gutierrez et al., 2021; Slifierz et al., 2015; Valeris-Chacin et al., 2021). Several studies have examined the impact of the pig microbiome on the immune response to vaccination (de Jong et al., 2020; Ruck et al., 2020), but the impact of vaccination on the microbiota had not been extensively analyzed, with a few studies focusing on gut microbiota (Guevarra et al., 2021; A. H. Kim et al., 2020; Leite et al., 2018). No information is yet available about the effect that specific stimulation of the immune system by vaccines could have on the nasal microbiota of the piglet.

Here, we report the effect of vaccination of sows against virulent *G. parasuis* on the nasal microbiota composition of their offspring. A reduction in the relative abundance of *G. parasuis* sequences was found in the samples from the piglets derived from vaccinated sows. In addition, other differences in the microbial composition were detected; especially, on the most relatively abundant bacterial taxa, as well as in inferred pathways, which were divergent between piglets born to vaccinated or non-vaccinated sows. Correlation Network Analysis (CNA) resulted in two complex networks with a different number of nodes and edges. The structure of the modules containing the most connected nodes in the co-occurrent ASVs control network was not conserved in the network from vaccinated sows.

RESULTS

Sequencing throughput stats

After the denoising and quality control steps, a total of 9,817 features were found in the 22 samples of the study. A total of 9,202,583 sequences were analyzed with a minimum ASVs frequency per sample of 211,733 and maximum of 649,315, being the mean 418,299. The ASVs had a mean length of 414 bp (range 236 - 444), where 98% of the sequences were 428 bp length. After processing, 73% of the raw sequences passed the quality-control steps and were used in the downstream analyses.

Sow vaccination decreased diversity in the nasal microbiota of the offspring

Richness and alpha diversity metrics (Observed Features, Simpson, Shannon, Faith phylogenetic diversity and Chao indexes) were calculated and compared between piglets born to vaccinated sows (vs-P) and to non-vaccinated sows (cs-P). Nasal microbiota from vs-P was significantly less diverse than cs-P when using Simpson diversity index and Simpson Evenness index (Figure 1). The rest of indexes showed the same tendency although they did not detect statistically significant differences between both groups.

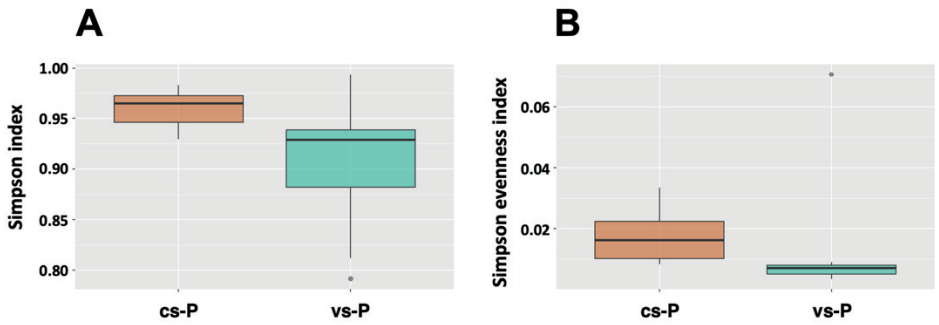

Figure 1. Alpha diversity measured as Simpson index and calculated as 1-D (**A**) and Simpson evenness index (**B**). Samples from nasal microbiota of 15-day-old piglets born to control unvaccinated sows (cs-P, orange boxes) and to vaccinated sows (vs-P, green boxes) with a vaccine against virulent *G. parasuis* were analyzed. These two indexes resulted statistically significant ($P < 0.05$).

The composition of the nasal microbiota evaluated through several beta diversity metrics showed a divergency between the vs-P and cs-P groups and this divergency was statistically significant when evaluated either quantitatively or qualitatively ($P < 0.001$, PERMANOVA test). The first axis in the PCoA (PC1) using Bray Curtis dissimilarity index, captured the highest amount of variation of the input data (53.14%; Figure 2A) and a high percentage of the differences were explained by the vaccination of the sows (R^2=51.47%; Adonis function). Spatial representation of the Jaccard distance (a metric that only calculates the presence or absence of the different ASVs in each sample) showed a 16.08 % of variation among the samples in the PC1 (Supplementary Figure S1), which was mainly explained by sow vaccination (R^2=15%, Adonis function). Similar results were also obtained with metrics that include phylogenetic relationships between the microorganisms. Thus, weighted (Figure 2B) and unweighted (Supplementary Figure S1) Unifrac showed PC1 with percentages of 44.12%, and 15.52%, respectively, which were in agreement with the percentage of explanation due to sow vaccination (R^2=41.2% for the weighted and R^2=11.5% for the unweighted distance). In all these analyses, sow vaccination explained a higher percentage of variation when the analysis included quantitative data.

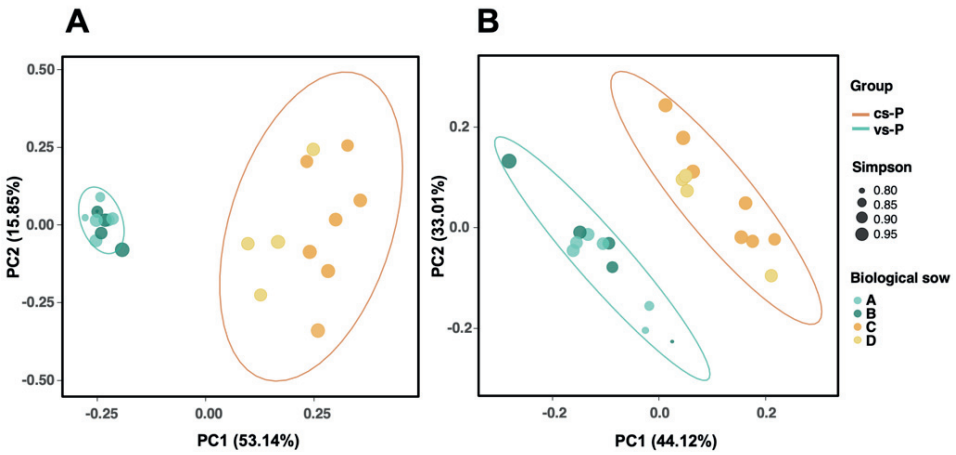

Figure 2. PCoA plots representing the beta diversity of the nasal microbiota of piglets from vaccinated (green) and control unvaccinated sows (orange). Bray Curtis distance matrix is represented in the left (**A**) and weighted Unifrac index on the right (**B**). Each sample is colored based on the biological sow, and the size of the symbol is proportional to the Simpson index (alpha diversity).

Taxonomical assignment and differential abundance analysis

The denoised sequences corresponded to 9,817 ASVs with different taxonomic assignment. Taxa assignation at the different levels showed a percentage of non-assigned taxa: 3.1% at phylum and 5.05% at genus. At phylum level, *Firmicutes*, *Proteobacteria* and *Actinobacteria* were the most relative abundant taxa in both cs-P and vs-P groups, while the ten most relatively abundant genera were *Enhydrobacter*, *SMB53*, *Rothia*, *Moraxella*, *Neisserieceae sp.*, *Lactobacillus, Clostridaceae sp.*, *Catenibacterium*, *Prevotella* and *Fusobacterium*. The top 30 most relatively abundant ASVs among all the samples from both groups were heterogenously distributed within each group and included 6 ASV classified as *Moraxella*, 3 as *Rothia*, 2 as *Prevotella*, and the rest of taxa represented by a unique ASV (Figure 3).

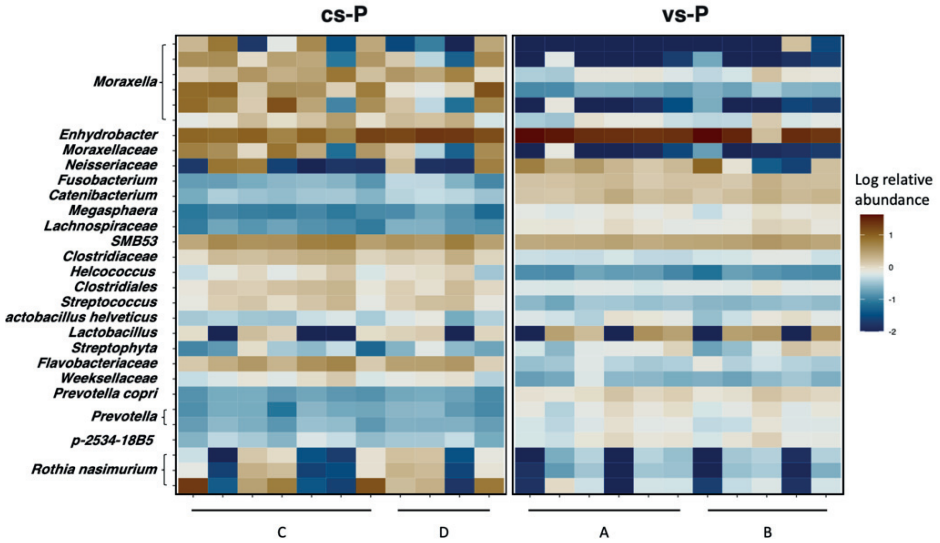

Figure 3. Heatmap representing the top 30 most relative abundant ASVs globally. The relative abundance (log10) of each ASV in the nasal microbiota of piglets from control sows (cs-P) and vaccinated sows (vs-P) is shown. The biological mothers are indicated in the bottom as A, B, C and D.

To unravel the differences in bacterial communities inhabiting the nasal microbiota from each group, we used two different approaches. First, the analysis of composition of microbiomes (ANCOM) showed 21 ASVs that differed between each study group (Supplementary Table S1). Eighteen of those ASVs were more abundant in the control group, and included *Moraxella*, *Staphylococcus*, *Prevotella* and *Actinobacillus*; all with relative abundances lower than 0.33%. ANCOM was also performed at different taxonomic levels and detected ten different phyla that were differentially abundant according to the study group. Seven out of these ten were increased in the vs-P group: *Tenericutes*, *SR1*, *Planctomycetes*, *Euryarcheota*, *Lentisphareae*, *TM7*, *Fibrobacteres*, *Fusobacteres*, *Verrucomicrobia*, *Spirochaetes*, and *Actinobacteria*. On the other hand, the phyla that were more abundant in the cs-P group were *GN02*, *Firmicutes*, and *Bacteroidetes*. At genus level, *Selenomonas*, *Megasphaera*, *Akkermansia*, and *Anaeroplasma* were associated with the vs-P group.

Second, the differential ranking *Songbird* method was used as a complementary method to infer taxa associated to either cs-P or vs-P group, showing a list of 1,509 ASVs. Once the low relatively abundant ASVs (less than 0.1% in either group) were removed, 186 ASVs showed

a statistically significant differential abundance, with 83 of them associated to the cs-P group and 103 ASVs associated to the vs-P group. The differential abundant ASVs included the 30 most abundant ASVs across all the groups, whose association to either vs-P or cs-P is depicted in Supplementary Figure S2. The 30 ASVs most associated to the vs-P group included ASVs classified as *Ruminococcaceae* (4 ASVs), *Megasphaera* (4 ASVs), *Paraprevotellaceae* (3 ASVs) and *Lachnospiraceae* (2 ASVs). In contrast, among the 30 most associated to the cs-P group, we found ASVs assigned to *Moraxella* (5 ASVs), *Moraxellaceae* (3 ASVs), *Ruminococcaceae* (3 ASVs), *Streptococcus* (3 ASVs), *Staphylococcus* genus (1 ASV), *Flavobacteriaceae* (2 ASVs) and *Rothia nasimurium* (2 ASVs) (Figure 4). In general, the differential ASVs classified within a given taxon seemed to predominantly be associated to one or the other group, e.g., *Moraxella* ASVs were associated with cs-P, while *Megasphaera* ASVs were associated with vs-P (Figure 4). Among the most relatively abundant ASVs (Supplementary Figure S2), twelve were shared with the most associated to each group (with high log-fold change value) corresponding to the following taxa: *Rothia nassimurium* (2 ASVs), *Prevotella copri*, *Flavobacteriaceae*, *Helcococcus*, *Lachnospiraceae*, *Megasphaera*, *Fusobacterium*, *Moraxellaceae* and *Moraxella* (3 ASVs) (Figure 4).

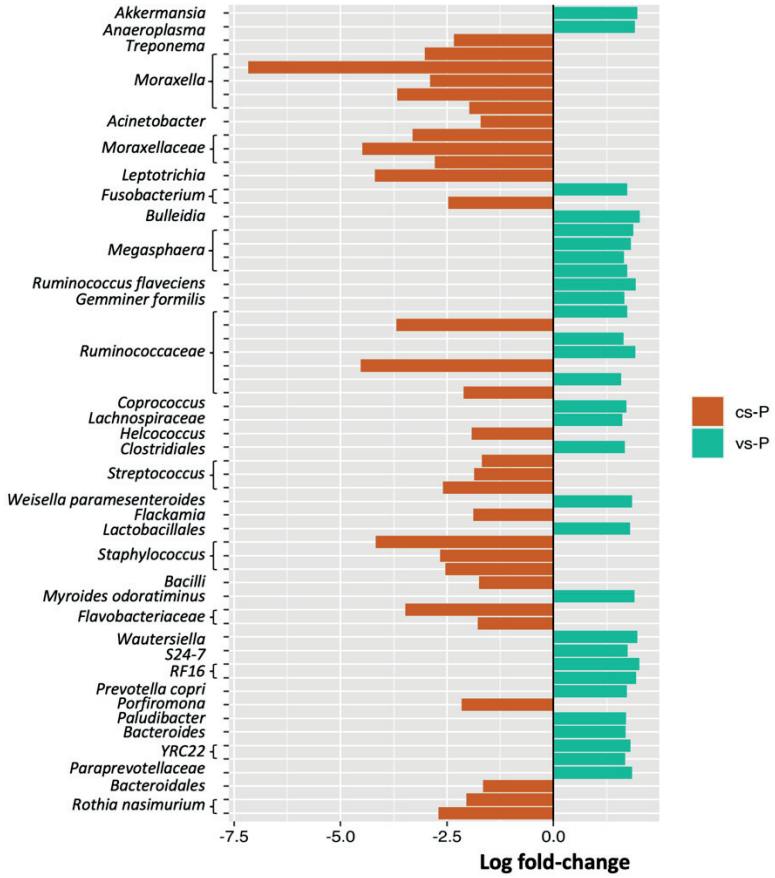

Figure 4. Differential abundant features between nasal microbiota composition of piglets from control (cs-P, orange bars) or vaccinated sows (vs-P, green bars). The association of the 30 features with the highest differential ranked coefficient estimated with *q2-songbird* is depicted (log fold-change).

Since the vaccine used targeted virulent strains of *G. parasuis*, we focused our analysis on the potential effect of this vaccine on this species. We found a total of ten different ASVs classified as *G. parasuis,* which in global showed lower relative abundance in the vs-P group than in the cs-P group (*G. parasuis* relative abundance: 0.058 % ± 0.029 for vs-P and 0.225 % ± 0.112 for cs-P). From these 10 *G. parasuis* ASVs, 5 were statistically associated to the control group, where three ASVs were exclusively found in the cs-P group. These findings were found by the Songbird differential rank analysis, but they were not detected using ANCOM.

Correlation Network Analysis

Correlation Network Analysis (CNA) at ASV level, resulted in two different undirected graphs when samples from the different groups were analyzed separately. The topology of the two graphs showed different characteristics. The cs-P network had 3,552 nodes with 1,678,545 edges and a network density of 0.266, while the vs-P network had 3,048 nodes with 1,324,390 edges and a network density of 0.285. On the other hand, the diameter of both networks was three.

The top ten most-connected nodes in the cs-P network ranged between 1,640 and 1,670 edges, and they were grouped in three different modules. Module 6 included ASVs from *Actinobacillus*, *Ruminococcus flavefaciens, Erysipelotrichaceae, Staphylococcus*, *Glaesserella parasuis* and *Filifactor* out of 76 total component nodes; module 8 (67 ASVs) included *WCHB1-25* and *Corynebacterium,* while module 13 (37 ASVs) included *Oscillospira*, and *Ruminococcus gnavus*. On the other hand, the most connected nodes in the vs-P were grouped in five different modules. Module 1 (124 ASVs) included the following most-connected taxa: *Lachnospiraceae* and *Clostridiales*; whereas *Coprococcus*, *Bacteroidales* and *Sphaerochaeta* taxa were highly-connected members of module 2 (91 ASVs). ASVs from *Moraxellaceae*, *Neisseriaceae* and *Moraxella* belonged to module 12 (37 ASVs), while the last two modules included *Oscillospira* in module 32 (23 ASVs) and *Lachnospiraceae* in module 41 (20 ASVs). None of the 10 most-connected nodes in the two networks was shared.

Among all the modules containing the ten most-connected nodes, module 6 from cs-P network had the highest number of these highly connected nodes, including an ASV classified as *G. parasuis* (Figure 5). When we compared the networks, we found that the ASVs from module 6 in the cs-P network, clustered differently in the vs-P network. From the 76 ASVs composing the module 6 from the cs-P network, 72 were found in 31 different modules in the vs-P network. Noteworthy, among these 31 modules, we found the module 23 containing an ASV classified as *G. parasuis,* reinforcing that the rearrangements in the community structure were due to vaccination against this pathogen. Five out of these 31 modules contained the top-ten most-connected nodes in the vs-P network (Figure 5). The remaining 4 nodes, assigned to *G. parasuis*, *Neisseria*,

Staphylococcus and *Actinobacillus porcitonsillarum*, were absent in vs-P network.

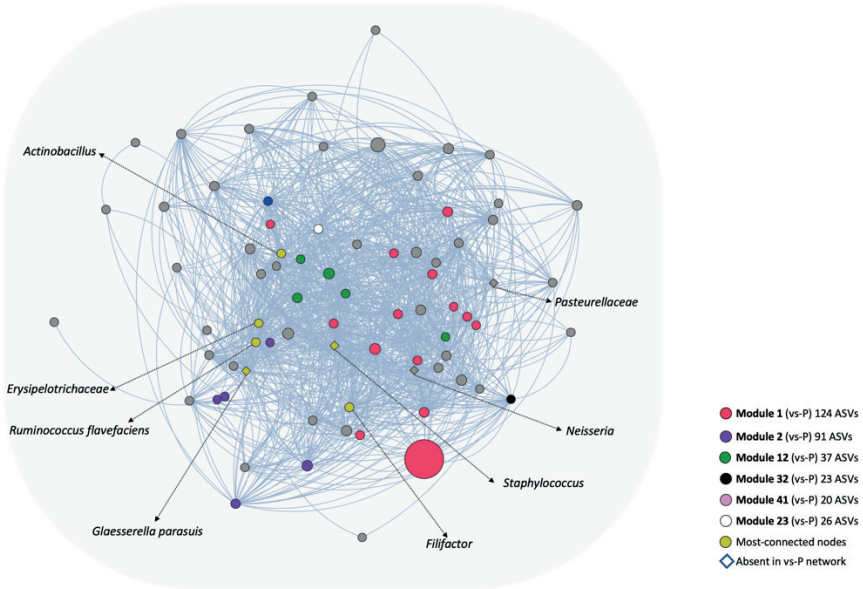

Figure 5. Subnetwork representing the module 6 of the co-occurrence network from the nasal microbiota of piglets from control sows (cs-P). Each node corresponds to ASVs and edges in the network represent the positive correlations between the different nodes. The nodes are colored based on the location of these ASVs in the network of the nasal microbiota composition of piglets from vaccinated sows (vs-P) modules. ASVs, in this module 6 from the cs-P network, that are present as the top ten most-connected nodes in the vs-P network, are represented in different colors regarding the modules they belong in the vs-P network: red, nodes in vs-P module 1; green, nodes in vs-P module 12; purple, nodes in vs-P module 2; black, node in vs-P module 32, and pink, for node in module 4. Yellow color highlights the most connected nodes in this module 6 from the cs-P network (6 nodes). The white node corresponds to an ASV classified as *Erysipelotrichaceae,* which is located in the same module as a *G. parasuis* ASV in the vs-P network. Diamond shaped nodes correspond to the four nodes that were absent in the vs-P network. Size of the nodes are proportional to their relative abundance, ranging from 0.0039% (*Neisseria*, one of the diamond shaped nodes) and 5.07% (*SMB53*, largest red-colored node).

Metagenome prediction and functional analysis inference

The metagenome was predicted from the 16S partial sequences in the two study groups to infer the potential functionality of the microbiota using the KEGG public database. PCA of the functional pathways showed a robust clustering of the samples by the group they belonged (Figure 6), in agreement with the composition (beta diversity) analysis (Figure 2) and suggesting that the microbial composition changes led to functional changes. Only 32 functional pathways were significantly different between the two study groups (Kruskall Wallis

test, *P* value with Bonferroni correction < 0.05, Supplementary Table S2), where 15 of them presented a two-fold change (Figure 7). The most relatively abundant pathways in the cs-P group were the isopropanol biosynthesis and L-tryptophan degradation to 2-amino-3-carboxymuconate semialdehyde, while the most relatively abundant in the vs-P group were L-arabinose degradation IV and the tetrahydromethanopterin biosynthesis. Pathways related with the biosynthesis with the peptidoglycan were in higher abundance in the cs-P group and with the biosynthesis of the LPS was higher in the vs-P group.

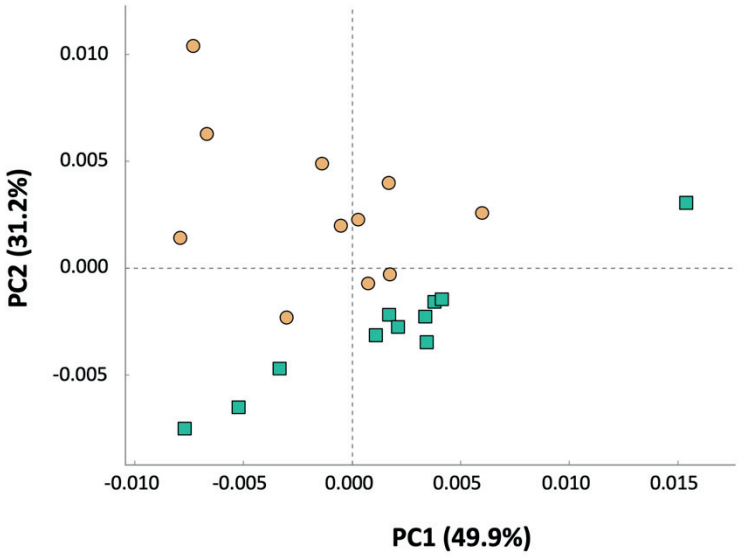

Figure 6. PCA of the functional pathway composition of each sample. Samples from piglets from vaccinated sows (vs-P group) are represented as green squares and samples from piglets from control sows (cs-P group) are represented as oranges circles.

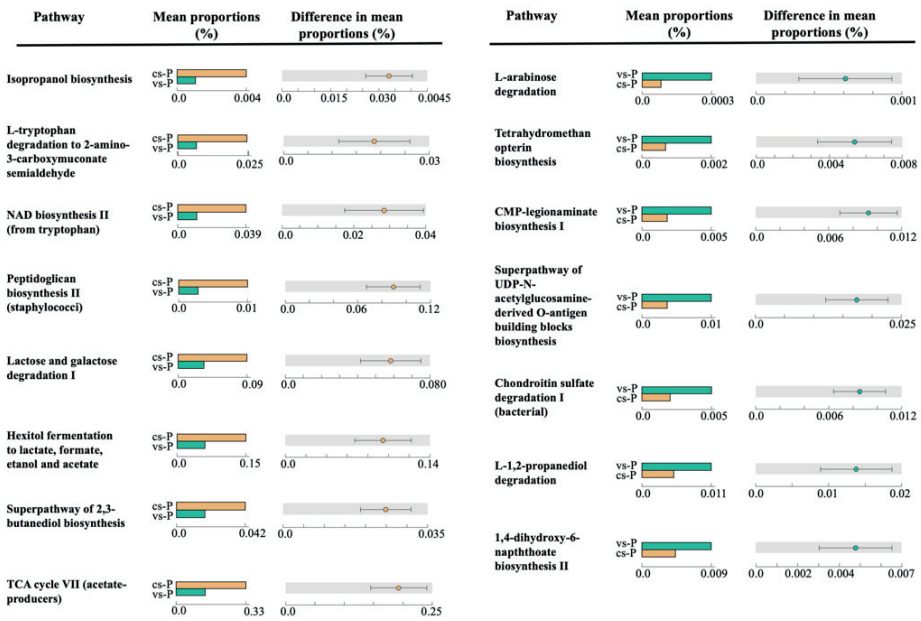

Figure 7. Predicted KEGG functional pathways using PICRUSt analysis. Most statistically significant differential pathways between the nasal microbiota of piglets from vaccinated (vs-P) and control sows (cs-P) are shown (Kruskal–Wallis *post-hoc* test with Bonferroni correction). Pathways more relatively abundant in the cs-P samples are shown on the left, and those more relatively abundant in the vs-P samples are shown on the right.

DISCUSSION

Vaccination against specific pathogens has been an extensive method to control infectious diseases for a long time now. Primary function of all vaccines is the stimulation of the immune system against specific antigens, while one of the main tasks of the microbiota is to contribute to the maturation of the immune system (Borey et al., 2021; Ciabattini et al., 2019; Lynn et al., 2021; Munyaka et al., 2020). Thus, the influence of the microbiota on the vaccine response has been more studied than the effect that the vaccines may have in the host microbiota and, in particular, in the microbiota of the progeny (Guevarra et al., 2021; Salgado et al., 2020). Here, we have shown that sow vaccination modified the nasal microbiota composition of their piglets with an effect extended beyond the targeted pathogen.

Vaccination reduces the shedding of the targeted pathogens (Leite et al., 2018). Accordingly, here we observed a reduction of the

relative abundance of all the *G. parasuis* ASVs after vaccination. Some of these ASVs classified as *G. parasuis* were statistically associated with the control group, while two of them were absent in piglets born to vaccinated sows. This reduction may be due not only to the decreased shedding from the mother but also by the maternal immunity transferred through the colostrum to the piglets as specific antibodies (Le Doare et al., 2018). As the vaccine used was designed against virulent strains of *G. parasuis*, it would be very interesting to determine if in fact, the virulent strains were specifically reduced in the piglets from vaccinated sows. Additional analyses, including shotgun sequencing metagenomics, may provide better resolution to study the different strains (variants) in the microbiota.

Sow vaccination also affected the general composition of the piglets' nasal microbiota. The nasal microbiota of the offspring from vaccinated sows showed a slight decrease in alpha-diversity. This was especially observed with the Simpson index (biased towards abundant features (Daly et al., 2018; B.-R. Kim et al., 2017)), indicating that the bacterial communities that are in relatively higher abundance in the nasal cavity of piglets drove the differences observed in our groups. In agreement, beta diversity analysis showed a higher percentage of explanation of the clustering in the indexes that include the quantity of the ASVs. Taken together, our findings indicate that vaccination of the mother did not change the main components of the nasal microbiota in the offspring but caused a clear disparity in the distribution of the taxa. In the differential abundance analysis, we observed that Songbird, a differential rank approach, discovered many ASVs that were not detected by ANCOM (1,509 ASVs detected by Songbird *vs* 21 ASVs by ANCOM) It was reported that ANCOM fails to infer differential microbes in some composition scenarios, where these are actually changing (Morton et al., 2019). The top ranked ASVs (most positive or negatively correlated to sow vaccination) within a given taxa tended to be associated to only one of the groups. Thus, ASVs from *Moraxella*, *Streptococcus*, *Staphylococcus* and *Rothia* were associated with cs-P, while ASVs classified as *Megasphaera*, *YRF22* and *RF16* were

associated with the vs-P group. However, this specific association of the ASVs from one taxon to one group was not observed in other ASVs, reinforcing the importance of detailed analyses at the ASV level. Strains within a given taxon can be heterogeneous, as demonstrated for *Glaesserella* (Mahmmod et al., 2020; Olvera et al., 2006) or *Moraxella* spp. nasal isolates (López-Serrano et al., 2020), and therefore they can play a different role in the microbiota.

Correlation Network Analysis has been a useful tool to identify global relationships between the microorganisms that compose the microbiota. Here, we observed that the network of piglets from vaccinated sows had lower complexity, demonstrated by the lower number of nodes, modules and edges, when compared to the control network. The structure of the modules containing the most connected nodes from the control network were not conserved in the structure shaped by the vaccination of the sows. Both the lower shedding of the pathogen by the mother, which in turn may produce a reduction of the pathogen in the piglet, and the effect of the maternal immunity transferred by the colostrum, might contribute to this rearrangement of the network topology.

The strong clustering between the two groups of piglets observed in the beta diversity analyses, resulted in a clear differential predicted metagenome, i.e., differential predicted functionality. Some of the metabolic functional pathways that were in higher abundance in the control group, such as isopropanol and L-tryptophan degradation are related to an immune suppressive status in mammals, as reported in the literature. On the other hand, none of the pathways in the vs-P group was related to any function of the immune system. The changes that we observed in the functionality may affect the health status or the immune response in the future, but this is difficult to assess and deserves further exploration. In general, the ASVs assigned to taxa of potential pathogens (*Staphylococcus*, *Neisseria*, *Streptococcus* and *Mycoplasmataceae*) were negatively correlated with the vs-P group, suggesting that the reduction of virulent strains of *G. parasuis* may contributed to drag out other associated pathogens in the nasal cavity (Blanco-Fuertes et al., 2021;

Correa-Fiz et al., 2016; Mahmmod et al., 2020). Moreover, potentially beneficial bacteria, such as *Prevotella*, were associated with the vs-P group. Members of the *Prevotella* genus have been associated to positive outcomes in pig production, including growth performance and immune response (Amat et al., 2020b; Correa-Fiz et al., 2019). Indeed, 122 from the 172 ASVs classified as *Prevotella* were associated with sow vaccination.

In summary, sow vaccination with a peptide directed against virulent *G. parasuis* influenced the nasal microbiota of the offspring, not only reducing the relative abundance of the targeted pathogen but also modifying the balances in general composition of the microbiota during at least 15 days of life. These changes in nasal microbiota composition are expected to affect its functionality.

MATERIALS AND METHODS

Study design and sampling

The experimental study was described previously by Lopez-Serrano et al (2021) and was performed under the approval of the Ethics Commission in Animal Experimentation of the Generalitat de Catalunya (Protocol number 9211) and was carried out in compliance with the ARRIVE guidelines. Briefly, four pregnant sows were included in the study based on low antibody levels against *G. parasuis*. Two sows were vaccinated intramuscularly with 2.5 ml of 100 µg/mL F4 (an antigen that is specific of virulent strains of *G. parasuis*) in combination with Carbopol 5984 EP polymer adjuvant (Lubrizol, Cleveland, OH, USA), while the other two sows remained unvaccinated and were used as control. Vaccination was performed at 32 and 12 days pre-farrowing. Newborn piglets were kept with their biological mother during their first hours of life (at least 12h). Twenty-four hours after delivery, only one sow from each group was maintained in the study, with biological piglets and cross-fostered piglets from the other sow from the same study group. Nasal swabs were taken from 11 piglets from vaccinated sows (vs-P) and 11 piglets from control sows (cs-P) at 15 days of life. Nasal swabs were

resuspended in 500 µl de PBS, vortexed for 30 seconds and stored at -80ºC until used for microbiota analysis.

DNA extraction and 16S rRNA gene amplicon sequencing

DNA was extracted from 200 µl of resuspended swabs using the Nucleospin Blood (Macherey-Nagel) kit. Quantity and quality assessment of the DNA was performed using BioDrop DUO (BioDrop Ltd). DNA samples were submitted to the Servei de Genòmica (Universitat Autònoma de Barcelona) for 16S rRNA amplicon sequencing using Illumina MiSeq pair-end 2×250 bp technology following the manufacturer instructions (MS-102–2003 MiSeq® Reagent Kit v2, 500 cycle). The region amplified covered the V3-V4 hypervariable regions (Johnson et al., 2019). The complete dataset from this study is available at the NCBI database, under SRA accession number PRJNA783272.

Microbiota analysis

Bioinformatics analysis was done using the Quantitative insights into microbial ecology (Qiime2) platform (Bolyen et al., 2019). Quality control step was done using *q2-dada2* plugin, where all the reads with a Phred quality-value under 20 and a length under of 234 bp in the forward read and 229 bp in the reverse read, were removed. Chimeric-reads check was also done and were removed for further analysis. In addition to the quality-control step, an alignment against the reference Greengenes (version 13.8) 16S rRNA gene database

(McDonald et al., 2012) was done to remove sequences not matching with an identity of 80% and a query length of 50%. Richness and alpha diversity analysis were done using Observed Features (in this case, Amplicon Sequence Variants, ASVs) (C. Shannon & Weaver, n.d.), Shannon index (C. Shannon & Weaver, n.d.), Chao index (Eren et al., 2012), Simpson Index and Simpson evenness (Grabchak et al., 2017). Different beta diversity metrics were used to assess the diversity across the samples, both qualitatively with Jaccard similarity coefficient (Jaccard, 1908) and quantitatively using Bray Curtis dissimilarity index (Bray & Curtis, 1957). Moreover, phylogenetic metrics weighted and

unweighted Unifrac (Lozupone & Knight, 2005) were calculated as well. These distance matrixes were used to perform Principal Coordinate Analysis (PCoA) using *core-metrics* plugin and a PERMANOVA test was done to analyze the clustering of the sample groups. To extract the percentage of variations explained by each metadata column, the Adonis function was performed on every distance matrix using *Vegan* (lab, 2019) package. Taxonomic assignation of each amplicon sequence variant was done using the Qiime2 classifier trained with the V3-V4 region from 16S gene and the Greengenes database (13.8 version) (McDonald et al., 2012). Differential abundant analysis was done using the analysis of composition of microbiomes (ANCOM) (Mandal et al., 2015) and the Songbird differential ranking (Morton et al., 2019) algorithms.

Metagenome prediction from the 16S rRNA sequencing was done using PICRUSt2 (Douglas et al., 2020). This software aligns the ASVs and place them in a tree (EPA-NG, GAPPA) with reference sequences and it infers the family genes and function pathways from KEGG (Kanehisa et al., 2017) orthologs and Enzyme Classification numbers. Together with the gene family copy number references, it predicts the gene content per ASV and with the abundance of each ASV in the study dataset it can determine the gene family abundance per sample. Mapping the gene family to pathways abundances was done using MinPath (Ye & Doak, 2009) integrated in the PICRUSt2 pipeline. Statistical Analysis of Microbial Profiles (STAMP) (Parks et al., 2014) software was used to visualize and perform statistical analysis over the predicted functional pathways outputted by PICRUSt2. Statistical analysis was based on a Kruskal Wallis test and Tukey-Kramer *post-hoc* test of the functional pathways of the different study groups were done using a Kruskal Wallis (Kruskal & Wallis, 1952) test and a Tukey-Kramer *post-hoc* test (Beyer, 1981). After a correction with Bonferroni *P* values under 0.05 were considered significant.

We built two separate networks from the ASVs of each group samples (cs-P and vs-P) Correlation Network Analysis (CNA) was performed using the Sparse Co-occurrence Network Investigation for Compositional data (SCNIC) integrated in the *q2-scnic* plugin from Qiime2 (Shaffer et al., 2020) software toolkit. All the samples that had less than 500 features and also all the features that had an average mean

less than 2 across all the samples, were filtered out. Pairwise correlations between all our ASVs in our dataset were calculated with *sparCC* (Friedman & Alm, 2012) algorithm. From that correlation table, we built our network with a minimum of R correlation-value cutoff of 0.35. Detection of modules was done by the summarization and clustering high connected nodes. Output files with nodes membership and edged connections were analyzed in Cytoscape (P. Shannon et al., 2003) software and extracted to tables to further data analysis to query ASVs and nodes. These tables were processed in Rstudio (Rstudio Team, n.d.) and merged with previous results using *tidyverse* package (Wickham et al., 2019).

All the plots and figures were done using R script language. Rstudio (Rstudio Team, n.d.) was used as a coding work environment. The packages used to build the plots and figures were specifically: *qiime2R (Bisanz, 2018)*, *ggplot2* (Wickham, 2009) and *tidyverse* (Wickham et al., 2019).

SUPPLEMENTARY MATERIAL

Supplementary Figures and Tables are available at Zenodo repository in this link:
https://zenodo.org/record/7892375#.ZFQumOxBz66

GENERAL DISCUSSION

Polyserositis is a common disease among young pigs, with higher incidence in the nursery period. This disease is mainly caused by *G. parasuis*, *M. hyorhinis* or *S. suis*. These bacteria, which become part of the healthy respiratory microbiota of the piglets soon after birth can, under different circumstances, spread and cause systemic disease (Aragon et al., 2019; Gottschalk & Segura, 2019; Pieters & Maes, 2019). The mechanisms by which these pathobionts spread and cause disease are not well understood but all of them share the final outcome: inflammation of the serous membranes.

Previous to the start of this Thesis, the role of the nasal microbiota in the context of the systemic disease produced by *G. parasuis* (Glässer's disease) was analyzed (Correa-Fiz et al 2016). In Study I of this Thesis, this concept was further investigated and expanded to the association of the nasal microbiota with *M. hyorhinis* polyserositis development, confirming previous information showing that a lower microbiota diversity in different niches is associated to disease predisposition (Kriss et al., 2018). Moreover, the nasal microbial composition described in this study was different from the one associated to Glässer's disease predisposition (Correa-Fiz et al., 2016), indicating that different bacterial communities may predispose to the animals to different infections. In fact, similar results have been found in the tonsil microbiota associated to *S. suis* disease predisposition (Fredriksen, Neila-Ibáñez, et al., 2022). It would be interesting to analyze if these findings in the tonsils are confirmed at the nasal cavity level. Further comparative studies to establish changes in the nasal microbiota correlated to each specific pathogen may provide a diagnostic tool to predict the risk of suffering the corresponding disease after weaning.

As the measures to control polyserositis in the farm include antibiotic treatment and vaccines, antibiotic applied either to sows or to piglets, and sow vaccination against virulent strains of *G. parasuis* were evaluated in Study II and III, respectively. Application of antibiotics influences microbial community composition by reducing the microbial diversity not only in the gut, but also in the upper respiratory tract of infants and adults (Kumpitsch et al., 2019). On the other hand, the

elimination of antibiotics administered during the first week of life produced an increase of the microbial diversity in the nose and promoted piglets' health (Correa-Fiz et al., 2019). In Study II, ceftiofur treatment triggered very sharp changes in the nasal microbiota at early times, probably indicating the effect of the antibiotic over an unstable microbiota. These observations reinforced the fact that the microbiota is not stable during the first weeks of life, highlighting the relevance of studying when and how the microbiota is established and reaches maturity. In addition, this longitudinal study expanded the knowledge on the dynamic changes in the nasal microbiota validating the importance of the age as one of the drivers influencing the microbiota composition as previously reported (H. B. Kim et al., 2011; Saladrigas-García et al., 2022; Slifierz et al., 2015). The nasal microbiota composition of the piglets was irreversibly affected when pregnant sows were treated. Sow treatment is sometimes performed in the farms to avoid the maternal transfer of pathogens to their offspring, since the sows are considered the main source of pathogens. Under those conditions, the maternal transfer of pathogens was indeed reduced, but the transfer of other members of the nasal microbiota was also affected. These changes in the nasal microbiota may represent a higher risk of disease for the piglets in the future. In addition, expanding this area of research to other respiratory pathogens would provide interesting data to improve the outcome of microbiota interventions.

As indicated above, the first weeks of life are crucial for the establishment of the microbiota, when the immune system is maturing, representing a "window of opportunity" to prevent microbiome and immune alterations (Berrington et al., 2014; Torow & Hornef, 2017). It is well known that the exposure to different environmental factors in early life, shapes the microbiota and the development of the immune system (Belkaid & Hand, 2014), which may dictate the outcome of infections, including those of respiratory origin. Hence, this may represent the perfect timing to induce changes that shape the microbiota to a healthier status. In this work, this window of opportunity was used to explore the possibility of modulating the nasal microbiota by the

inoculation of putative beneficial bacteria within the first 24 hours of life. This work demonstrates for the first time that the use of nasal putative probiotics is safe in newborn piglets and can modulate the nasal microbiota of the animals. The use of these colonizers seems to be specially interesting for the restoration of the nasal microbiota after antibiotic treatment. Here, this fact was observed in the piglets born to treated sows, but it is foreseen that the inoculation of colonizers would be useful in treated piglets as well, taking into consideration the timing to avoid the effect of the antibiotic. Importantly, the approach explored in this thesis can be used to support piglet health to reduce the use of antimicrobials in swine production, and therefore, reduce the probability of antimicrobial resistance occurrence. The promising knowledge generated by the use of the selected colonizers should be expanded by testing other combinations of beneficial bacteria and in different conditions, such as co-infections of several pathogens.

Vaccination is also a good alternative to antimicrobial use. Vaccination against a specific pathobiont applied on sows reduced the nasal colonization of piglets by the pathogen but also shaped the microbiota composition. This intervention affects the establishment of the microbiota and may have a long-term effect on the health of the animals. In this sense, vaccination against other pathogens could also induce changes leading to valuable results that should be explored. In addition, inoculation of piglets with beneficial colonizers in combination with sow vaccination may represent a better strategy than the combination with antibiotic treatment of the sows. Vaccination does not contribute to the risk of emergence of antimicrobial resistances and is more specific against pathogens.

While most of the microbiome studies focused on the composition (What is there?) recently this research area expanded to include the functional profiling to unravel the genes and metabolic pathways (What are they doing?) (Ottman et al., 2012). Functional profiling can be inferred directly from the shotgun metagenomic sequences by the analysis of the presence and the quantification of gene families and their metabolic products. These analyses are important to

assess the impact of the different communities since differences in composition not always translate into different functionalities. This Thesis has shown that a broad spectrum antibiotic treatment produced drastic changes in bacterial composition that were not observed at functional level, indicating that there is redundancy and some metabolic functions can be performed by different taxa (Eng & Borenstein, 2018). Surprisingly, vaccination induced evident changes that were comparable at both composition and functional levels. The elimination (or reduction) of the specific pathogen by vaccination seemed to produce new networks that translate into new functions.

While the use of metatranscriptomics and meta-metabolomics can give more realistic results of the actual functions in the bacterial community (Ottman et al., 2012), the biological meaning of all these changes and the impact on the host are difficult to assess, in particular immunological parameters that could be associated to health.

The rapid expansion of the microbiome research over the past two decades was mainly due to the next generation sequencing techniques that emerged: amplicon sequencing (16S) and WGS metagenomics. These two innovative approaches revolutionized the field of microbiome research circumventing several limitations of previous studies. In this Thesis, both methods were used to uncover the effect of different interventions on the nasal microbiota of piglets. The tools develop for microbiome analyses are continuously evolving through worldwide collaborative work, which represents a challenge since it requires continuous updating. Hence, throughout this Thesis, the *in silico* analyses were adapted to these improvements. Every step is critical and may have a huge impact in the downstream analysis yielding different results. The analysis of amplicon sequence data typically begins with the discrimination of the different sequences. This has been typically done by clustering together the different sequences that show more than 97% of similarity (OTUs), which will be later taxonomically classified. This is a critical step, affecting all analyses. Hence, the appearance of a new approach that differentiated sequences with as little as one different nucleotide (ASVs), represented a groundbreaking milestone for analyzing bacterial communities (Amir et al., 2017). Several studies

pointed out that OTU clustering have less resolution for the taxonomical identification in 16S studies compared to the ASVs method, and therefore, this innovative approach was adopted in this Thesis to perform the analyses at the finest possible level. Recently, the risk of artificially splitting single bacterial individuals into multiple different ones using this ASV method has arised (Schloss, 2021), evidencing that all methods have their caveats that need to be considered when arriving to conclusions.

As already stated, the microbiota has a relevant role in health and disease. However, microbiota studies are complex and confounding factors may be difficult to control. A correct design of the studies can help in the control of some factors (e.g., same environmental conditions and diet for the groups to be compared), however others cannot be controlled, such as the inter-individual variability. Therefore, the inclusion of a significant number of animals in the studies is needed to find representative results. In addition, to study the relationship between the nasal microbiota and the predisposition to develop disease, the incorporation of more farms to the analysis may reduce the potential confounding effect of the different environments and probably reveal more insights. Moreover, the use of shotgun metagenomic sequencing data can provide genomic details, including relevant information on virulence-associated or antimicrobial-resistance genes, allowing a wider vision of the whole microbial communities.

The microbiota comprises all members living in a particular environment. In this thesis, we focused only on Bacteria, but Archaea, Fungi, Algae and Protists are considered active members of the microbiota and deserve deeper investigation since they might be affected by the different factors studied in this Thesis. Likewise, future studies should focus on deciphering whether the changes within nasal environment described along this Thesis, may be also extended to other ecological niches of the piglets' microbiome. In fact, several studies showed that gut microbiota promotes defense against viral respiratory infections while others reported potential mechanisms through which respiratory diseases can impact on gut microbiota (Sencio et al., 2021). suggesting a connection between them. This respiratory-digestive axis would open opportunities to work in the search of common therapeutic and preventive approaches.

Overall, this Thesis has expanded the knowledge on the role of nasal microbiota in polyserositis in nursery pigs and has paved the way for control strategies alternative to the classic antibiotic treatment.

CONCLUSIONS

1. The nasal microbiota of weaning piglets from farms with polyserositis cases caused by *M. hyorhinis* showed lower diversity and different composition compared to age-matched piglets from farms with good health status. These results were reinforced by the presence of the clinical-associated *M. hyorhinis* ASVs exclusively in the nasal microbiota of piglets from farms with *M. hyorhinis*-associated disease.

2. Different changes in the nasal microbiota composition were observed in weaning piglets from farms with polyserositis caused by *M. hyorhinis* or *G. parasuis*. These changes might facilitate invasion and the subsequent development of the systemic infections by *M. hyorhinis* or *G. parasuis*.

3. Ceftiofur treatment destabilized the nasal microbiota of piglets, with a longer-term effect when it was administered to the sow instead of directly to the piglet.

4. The usage of ceftiofur favored the selection of higher number of antimicrobial resistance genes in piglets or the appearance of different resistance genes to Beta-lactams depending to whom is administered.

5. The changes observed in the piglet's nasal microbiota due to the ceftiofur administration were not as evident in the functional profile of the microbiome, suggesting that different microbiota compositions can provide similar functions.

6. The effect of the sow antibiotic treatment on piglets' nasal microbiota was partially reverted by inoculating at birth a pool of nasal colonizers. This combined intervention reduced the clinical signs in the piglets, representing a strategy to improve the pig's health by using a non-invasive alternative to antibiotics.

7. Sow vaccination with a peptide directed against virulent *G. parasuis* shaped the nasal microbiota of the offspring, reducing the relative abundance of the targeted and other pathogens while increasing potential beneficial taxa. In addition, the vaccination also modified the co-occurrence network topology of the microbiota.

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