Exploiting the tonsil microbiota to prevent *Streptococcus suis* infections

Isabela M. Fernandes de Oliveira

Propositions

1. The tonsil is the main route of entry into the body for pathogenic *Streptococcus suis* (this thesis).
2. Commensal *S. suis* strains compete for the same oropharyngeal niche of disease-associated *S. suis* strains reducing the risk of systemic disease. (this thesis)
3. The publication of unexpected results are as essential as successful results.
4. Humanity's long-term health depends on the microbiome diversity.
5. Governments should ban the early weaning of piglets.
6. Digital disinformation is stopping society from being critical.

Propositions belonging to the thesis, entitled

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Thesis

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Dedico ao meu pai e a minha irmã

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Jessie Isabelle Price

I

General Introduction

The threat of infectious diseases in swine production and spread of antimicrobial resistance in the environment are global problems. The acquisition of resistance has been partly driven by the overuse of antimicrobials in clinical and agricultural settings, which limits preventative or curative treatment options. Common porcine disease-associated bacteria such as *Streptococcus suis* are among the main causes of antibiotic use in pig production. Because of lack of cross-protective vaccines, increasing antibiotic resistance and endemic carriage in pig herds worldwide, alternative effective control measures to avoid *S. suis* infections are highly important for animal health and welfare in the pig production industry. The aim of this thesis was to generate an extensive culture collection of isolates from the tonsil microbiota which is the main host habitat for *S. suis* and explore the potential to develop probiotic interventions to antagonise this pathogen. Concurrently we explored the untapped potential of the tonsil microbiota as a source of novel antimicrobial natural products through the *in vitro* screening and genome mining of hundreds of cultured isolates. The thesis also contributed to the understanding of *S. suis* ecology and pathobiology.

Pork industry and *Streptococcus suis* disease

In animal food production, pork meat production was estimated to be 105 million metric tons worldwide in 2021 (USDA report, October 12, 2022) (1, 2). Global consumption of pork is projected to increase to about 131 metric kilotons by 2027. Pork is the most popular and widely consumed meat source worldwide, with majority in Europe and Southeast Asia. According to Food and Agricultural Organization (FAO), there are over 752 million of pigs worldwide of which 50% are in China and the rest roughly divided between Europe and the U.S (3) (Figure 1). The anticipated growth of the pig population has fuelled increasing concerns for food safety and animal welfare by farmers, veterinarians and consumers. Pigs are also important both for biomedical research, because their anatomy and physiology is similar to humans.

In large commercial farms, infection with the Gram-positive bacterium *Streptococcus suis* is one of the main reasons to prescribe antibiotics in the weaning period (4). Weaned and suckling piglets receive over 80% of the antibiotics used in the pork industry, depending on pig husbandry system and country-specific regulations (5). *S. suis* causes severe infections that may lead to meningitis, septicaemia, and arthritis. *S. suis* is also an emerging zoonotic pathogen that causes the same diseases occurring in piglets (6). Most human cases have been reported in Southeast Asia which is thought to be due to differences in farming systems, more human exposure to undercooked pork or pork products as well as culinary habits.

Epidemiology of *S. suis* was originally based on 35 antigenically distinct capsular polysaccharide serotypes (1–34, 1/2) (7). However serotypes (20, 22, 26, 32, 33, and 34) were recently assigned to closely related species, bringing the total to 29 serotypes (8). Non-typable *S. suis* isolates are also frequently isolated, mainly from healthy piglets. Most porcine *S. suis* infections worldwide are caused by a relatively small number of serotypes such as serotype 2, 9, 3, 1/2, and 7 (7, 9). Serotype 2 strains are mostly associated with disease in both pigs and humans worldwide including Europe, some Asian countries such as Cambodia, China, Hong Kong, Japan, Thailand, and Vietnam, as well as Africa and Argentina (9) (Figure 1). Isolates associated with disease have smaller genomes and contain substantially fewer genes than non-clinical isolates which are more likely to encode key virulence factors (10).

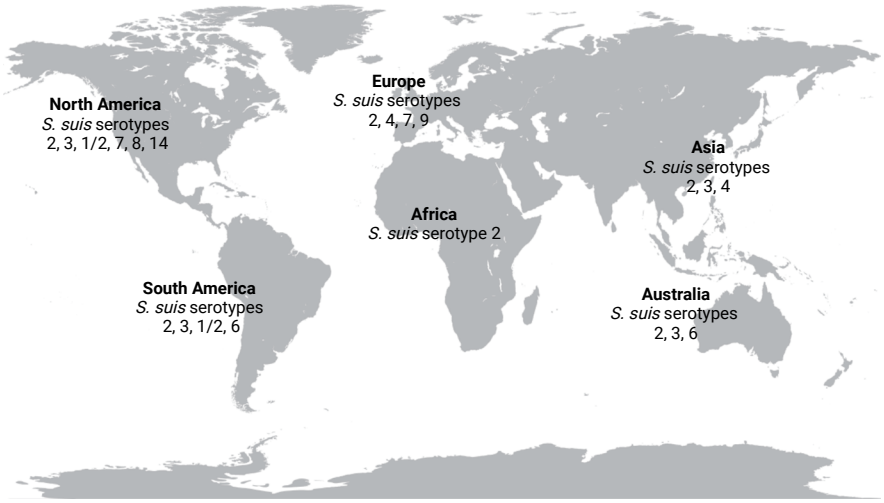

Figure 1. Global distribution of *S. suis* serotypes. Figure adapted from (9, 11).

The bacterial capsule enables *S. suis* isolates to avoid phagocytosis and complement mediated killing from the host immune system leading to severe systemic diseases. The capsules of some *S. suis* serotypes (e.g. 2 and 14) contain sialic acid. Sialylation of the capsule has been associated with immune evasion due to binding of sialic acid containing moieties to the host family of sialic-acid-binding immunoglobulin-like lectins (Siglecs), and these binding events block neutrophil and macrophage activation, and suppress platelet activation (12, 13).

Capsule production is one of the ca. 100 putative virulence factors that have been proposed to be involved in infection and/or pathogenicity of *S. suis*. Proposed *S. suis* virulence factors include surface-associated or secreted components, regulatory genes and metabolic pathways. At least 37 of these factors have been claimed as being 'essential' for virulence for some strains based on experimental animal models or clinical condition (9, 14). However, redundancies and contradictions have been reported for most *S. suis* virulence factors, because of huge genetic diversity of *S. suis* isolates including virulent strains (9). Virulence-associated factors were classified into four categories based on their properties and functions, including surface/secreted elements, enzymes/proteases, transcription factors/regulatory systems, and others transporters/secretion systems (6). Around 26 zoonotic virulence factors have also been proposed based on their higher prevalence in human isolates compared to pig isolates (15).

S. suis colonises the upper respiratory tract (particularly the palatine tonsils of the soft palate) of pigs (16, 17), and can be isolated from the birth canal. *S. suis* is also occasionally found in low numbers in the intestine. As a species *S. suis* is known to comprise non-disease associated isolates and pathogenic isolates defined by their isolation from blood or brain of diseased animals after necropsy (10). The non-disease associated strains of *S. suis* isolates are considered to have a commensal 'lifestyle' and be less virulent than the pathogenic isolates. Recent studies have shown *S. suis* to be relatively abundant proportion of the tonsil -associated microbiota (about 6 to 10%) on all farms in all European countries surveyed, suggesting that carriage does not necessarily lead to disease (18, 19). The factors leading to invasive disease are not fully understood although co-infections with viruses such as swine influenza, or PRRS and stresses caused by abrupt weaning are thought to increase host susceptibility to infection. *S. suis* may cross the epithelium in the upper respiratory tract to enter the body although invasion has been shown to be low in various cell lines *in vitro* (20, 21). However it is possible that adherent *S. suis* can damage the epithelium via the secretion of suilysin, a cholesterol-binding pore forming toxin and translocate into the body (22). The palatine tonsils (discussed below) is considered to be the main route of entry into the body; this has been hypothesized because of the relatively high abundance of *S. suis* on the tonsil, and the biological role of the tonsil in sampling antigens and intact, live bacteria for eliciting mucosal immune responses. The intestine and respiratory tract have also been proposed as routes of entry into the body. (23-25).

Soft palate tonsil, microbiota and colonization resistance

Pigs have five tonsils named lingual, paraepiglottic, pharyngeal, tubal, and palatine tonsil (or tonsil of the soft palate). The palatine tonsils, located at the ventral side of

the soft palate are the largest and most developed porcine tonsils, around 5cm long and 3cm wide (26). The porcine palatine tonsils are located at the portal of entry of the gastrointestinal and respiratory tracts and contain mucosal-associated lymphoid tissue for eliciting innate, cellular and humoral immunity at mucosal surfaces, particularly the upper respiratory tract (27). Long and numerous crypt invaginations within the tonsillar lymphoid tissue increase the contact area between the external environment and internal layers of lymphatic tissue (28). The tonsillar stratified epithelium covering the oropharyngeal surface is continuous towards the crypts opening which consist of non-keratinised epithelial cells and numerous intraepithelial immune cells (27). Tonsillar lymphoid follicles become visible under the mucosal epithelium of the soft palate tonsils around 7 days after birth; their development may start at day one after birth and follicle dimensions gradually increase with age (29, 30). The lymphoid follicles are considered to be the activation sites of B and T cells (30).

As a secondary lymphoid tissue with a strategic position at the entry portals of gastrointestinal- and respiratory tract, the tonsils form the first immune line of defence against the invasion of pathogens in the upper respiratory tract. The palatine tonsil immune barrier is composed by subsets of lymphoid and myeloid cells consisting of monocytes, dendritic cells and lymphocytes (31). Histological analysis of tonsil sections demonstrated that stained bacteria were mostly restricted to areas close to the crypt lumen (32). Antigens from the oral and nasal cavity may enter the organism through the tonsils via the crypts. Wilson et al, performed a immunohistological study with gnotobiotic piglets to identify cells associated with bacteria in the tonsils of animals diagnosed with *S. suis* infections (32). In these animals, stained bacteria were closely associated with cells staining positive for markers of the myeloid cell lineage (32). Expression of CD16 and CD163 on the tonsillar leukocytes suggested an association with mature macrophages. *S. suis* was found closely associated with cells expressing combinations of the markers CD172, MHC class II, CD11b, CD16, CD14 and CD163 (32).

The immunologically active tonsils are populated by a large microbial community. The development of the tonsillar microbiome is a gradual and successional process with a significant change in composition after weaning (16). Relatively, little is known about the development of the core tonsil microbiota composition; some members appear to be shared with the skin, the teats and vaginal microbiome. Tonsillar microbiota composition is known to vary between litters over the following first weeks due to sow-dependent factors and in particular the transition from suckling to weaning (16, 33). Young pigs were shown to be mostly colonized by members of the *Actinobacillus*/*Haemophilus*, *Streptococcus, Moraxella*, and *Porphyromonas*

genera, with some groups being transitory and more abundant at specific times such as *Staphylococcaceae* in newborns and *Fusobacteriaceae* and *Leptotrichiaceae* at week 2 and 3 post-partum (16, 33). *Rothia*, *Neisseria, Alloprevotella, and Acineto- bacter* were reported in less amount but significantly abundant during the development of piglets (>3% relative abundance) (33).

A critical function of microbiota is to prevent the overgrowth of potential pathogens under homeostatic conditions using numerous strategies to promote protection via mechanisms collectively known as colonization resistance. In general, colonisation resistance pertains to the antagonism of pathogens through direct mechanisms such as production of antimicrobial compounds, or indirectly by stimulating host innate immunity. Direct mechanisms of colonization resistance are characterized by the ability of the commensal microbiota to restrict external microbial colonization or to prevent, via production of antimicrobial factors, pathogenic overgrowth of original microbial members. Indirect mechanisms of colonization resistance are characterized by commensal microbiota dependence on host-derived factors in order to provide protection against an exogenous pathogen (34).

The underlying mechanisms by which the members of the resident microbiota confer resistance to pathogen replication and infection include nutrient depletion, microbiota-induced stimulation of innate and adaptive immune responses, and direct inhibition of pathogen growth by antimicrobials such as bacteriocins or natural products (Figure 2). It has been suggested that bacteria from the same niche share a common core of genes that are essential to scavenge local nutrient resources and to handle ecological constraints and microbial competition (35). The microbiota is known to utilize numerous nutrients not absorbed by the host's intestine. It has been reported that severe calorie restricted diet can lead to significant decreases in total bacterial abundance and a lessening of colonization resistance, increasing the chances of pathobionts growth during the restructure of the microbiota (36). Efficient competition for nutrients by commensal microbes can establish colonization resistance by depleting all potentially available nutrient niches which support growth of pathogens.

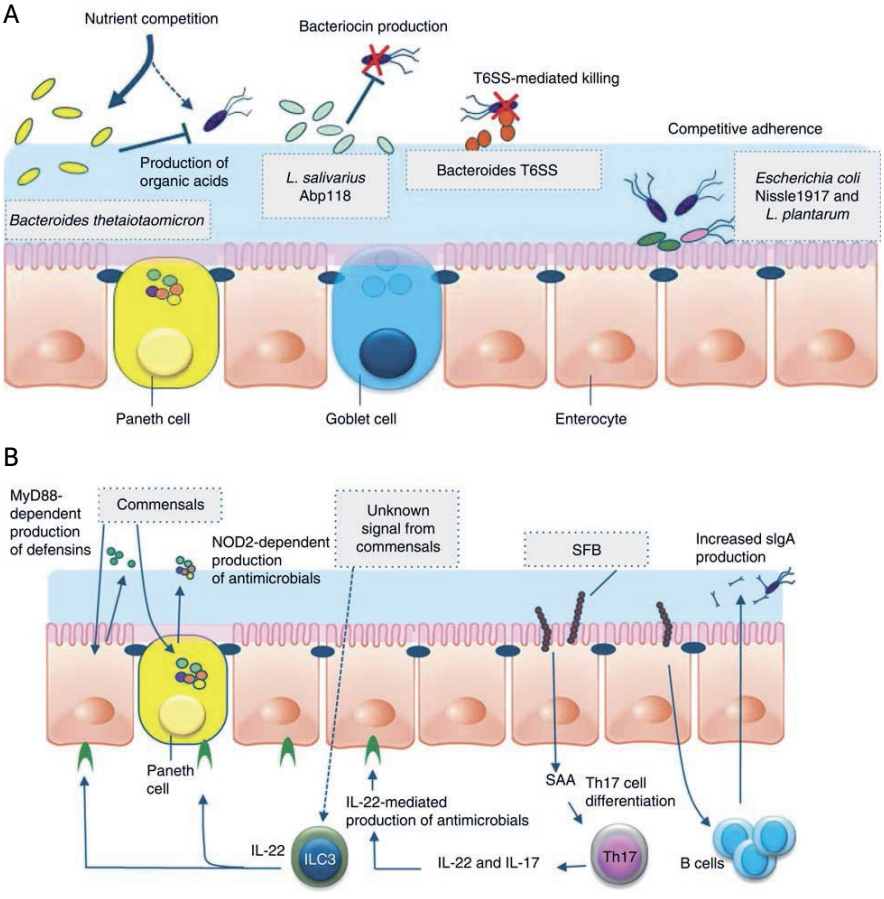

Figure 2: Microbiota-mediated colonization resistance by direct and indirect mechanisms. Microbiota carry out food fermentation and production of antimicrobials inhibiting the growth of pathogens. **(A)** Some bacteria directly inhibit intestinal pathogens by competing for nutrients or by inducing the production of inhibitory substances which may support pathogen growth; representative taxa are named in the illustration. Pathogens and pathobionts can use dietary and host- derived nutrients and metabolites to support growth as well as virulence. Some bacterial species may inhibit colonization by pathogens through a combination of direct and indirect mechanisms (for example, *B. thetaiotaomicron*, or may require other bacteria to carry out antagonistic effects. **(B)** Apart from these direct mechanisms of competition, the resident microbiota stimulates host innate immunity which protects against pathogen colonisation and invasion via recognition of bacteria by host pattern recognition receptors that activate immune defences and protect against infection. For example, sensing of commensal bacteria via IL-22 receptor stimulates innate lymphoid cells (ILC) to secrete IL-22, which stimulates intestinal epithelial cells to increase production of fucosyltransferase, mucin and

antimicrobials including Reg3 proteins. Segmented filamentous bacteria (SFB) associate with the ileum epithelium and stimulate maturation of the B- and T-cell compartments leading to enhanced IgA production by B cells and T helper 17 (Th17) cell differentiation. Figure and legend adapted from reference (37).

Microbiota can inhibit pathogen colonization indirectly, by stimulating host innate immunity. Common molecular pattens or structures on bacteria such as lipo-polysaccharide (LPS) on Gram-negative bacteria are recognised by host pattern recognition receptors. This triggers host innate defences such as production of epithelial defensins, mucin secreted by goblet cells in epithelial cells (37). This concept lies behind the notion that administration of commensal microbiota members and/or their metabolites can be used to prevent and even reduce infections that are caused by pathogenic bacteria, by application of so called microbiome based therapies (Figure 3) (37). The understanding (and possibly manipulation) on protective microbiota compositions and their bioactive metabolites may direct beneficially impact host immune defences and physiology, as well as bring opportunities for the development of novel therapeutics approaches to control disease-associated microbes.

Genomics and culturomics: two complementary approaches to predict and investigate the functional potential of the tonsil microbiota

The advancements of "omics" techniques have generated large amounts of publicly available data which help to increase systems-level understanding of different ecosystems. Omics approaches facilitate studies of genes, regulatory elements and noncoding sequences (genomics and metagenomics), messenger RNA and gene expression (transcriptomics), protein (proteomics), metabolites and metabolic networks (metabolomics). Omics applications are now frequently incorporated into the methodologies of biological researchers (39). The resulting data can be used to predict ecological interactions and functions within the microbiome, interpretation and integration of information at microbial (metabolic) systems level is still challenging. Genomics analysis identify and describe genes and their encoded proteins and how these have evolved based on sequence information and annotations of genomic sequences is one of the "omics" approaches (39). These analyses, can assist in the identification of genes with specific metabolic functions or genes involved in virulence or resistance to antimicrobials, in conserved or variable regions of the genome, as well as the mining for prospective metabolites and natural products. Functional annotations of genes and whole genome sequences obtained from microbiota consortia, or metagenomes, are crucial to discover the genetic potential of the microbiota or individual species and to

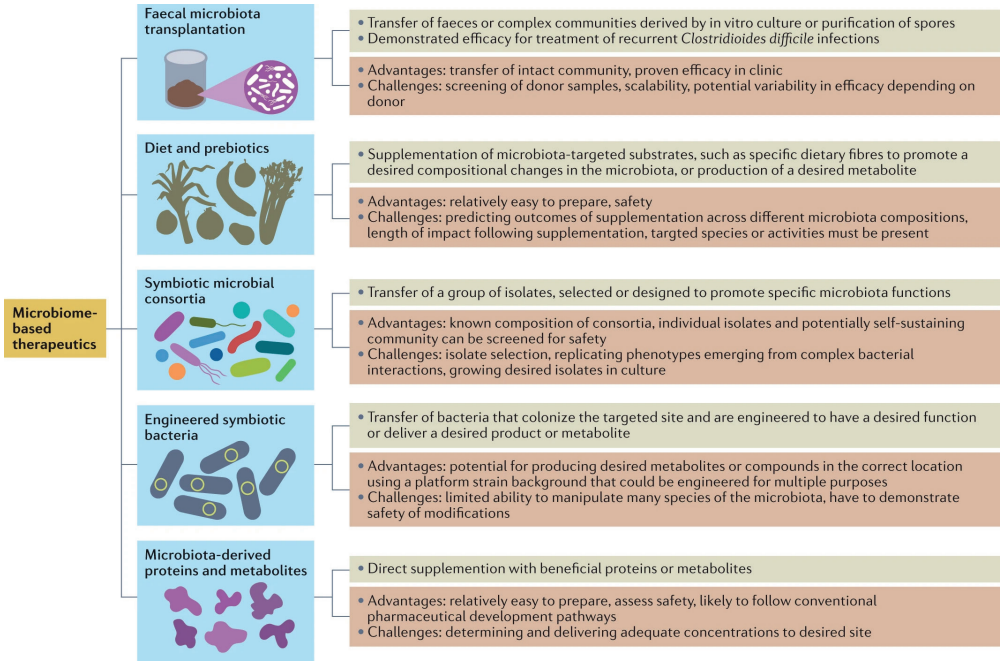

Figure 3: Microbiome based therapeutics are gaining increasing attention of the scientific community. The main approaches are faecal microbiota transplantation, supplementation with prebiotics, transfer of symbiotic microbial consortia or engineered strains, and directly providing microbiota-derived proteins and metabolites. Figure adapted from (38) with permission of the Springer Nature, Copyright Clearance Center.

understanding the ecological diversity of microbiota (40). (Meta)genomic analysis commonly include two steps; assembly of short or long DNA sequence fragments, and gene or genome annotation (41). Genome assembly basically puts together all (meta)genome sample fragments to form a set of contiguous, optimally overlapping sequence fragments and contigs that represent the collective DNA from a single microorganism or all microbes present in the sample, including viruses, bacteria, fungi and host DNA. Genome annotations describe structural and functional information from the genome, in particular, the genes and their products (42).

However, sequencing and *in silico* (meta) genomic analyses cannot easily predict the microbial viability and their functionality; moreover, results may be biased by the use of traditional but suboptimal markers, bias in databases (e.g. clinically relevant and therefore often studied bacteria are overrepresented) and statistical

approaches (43). Integration of culture-dependent methods with genomic data may provide a more coherent picture of bacterial metabolism, gene expression and identification of novel species, for instance by fatty acid analysis of proliferating bacteria. Screening and cultivation to obtain pure cultures of prokaryotes is essential to fully characterise different species, and their metabolic pathways can be validated *in vitro.* Microbial culturomics has emerged as a high-throughput approach to culture numerous species, and culturing species is crucial for further *in vitro* characterization or host interaction studies (43). Culturomics approaches consist of different stages of laboratory activities, from the selection of the samples and establishment of different culture conditions and identification of bacterial species towards *in vitro* tests and screenings (44). A large collection of 7,758 gut bacterial isolates paired with 3,632 genome sequences and longitudinal multi-omics data showed that genomic diversification can be used to infer ecological and evolutionary dynamics of microbiota as well as *in vivo* selection pressures for specific strains within individuals (45). This highlights the importance of the use of culturomics as a complementary approach to enrich current databases with characteristics of novel species.

Extensive reviews have listed substantially increased numbers of isolated and cultured prokaryotic species from different human sites, from 2170 species in 2015 to 3253 species in 2021. More than 60% of reported novel species have been obtained by culturomics (46-48). Culturomics studies have also enlarged the spectrum of prokaryotes associated with infectious diseases (49). Recently methods to obtain high numbers of near-single colonies of bacterial cells from millions of picoliter droplets via a microfluidic platform have been developed to complement the conventional approaches (50) (51). Even though culture dependent approaches are essential for functional and mechanistic studies in different animals and model organisms most studies have focused on the human gut (43, 45). Microbial isolate and genome collections from different animals and animal body sites are still limited, which hampers for example studies into strain-level diversity of disease-associated bacteria such as *S. suis* (15, 52)

Discovery of biosynthetic gene clusters (BGCs) predicted to produce bioactive natural product

Biosynthetic gene clusters (BGCs) are clusters of two or more genes that are involved in a single metabolic pathway for the production of a specific metabolite(s) (53). The natural-product BGCs often carry core biosynthetic genes in addition to genes involved in transport, regulatory elements, and genes that mediate host resistance to the molecular products they encode (54, 55). Global analysis of 1,154 complete bacterial genomes revealed an impressive diversity of BGCs (of which the

vast majority is yet uncharacterized) in the prokaryotic tree of life. This highlights the importance of natural product biosynthesis as a defining feature of bacterial metabolism (55). Functional analysis of BGCs is often challenging because their expression is highly dependent on complex regulatory networks based on biotic and abiotic stimuli from the external environment and their natural habitat (56). BGCs are not often expressed under standard laboratory growth conditions, and a range of strategies have been developed to activate their expression and identify the associated metabolic products. The high number and diversity of BGCs detected among closely related species from different microbiomes suggests the metabolites produced have important ecological roles (53).

BGCs have been classified into major structure-based classes: 'Alkaloid', 'Non-ribosomal Peptide (NRP)', 'Polyketide' (PK), 'Ribosomally synthesised and Post-translationally modified Peptide (RiPP)', 'Saccharide', 'Terpene', and 'Other' (57). Based on their biological function, the most prominent classes of the natural products are the PKs, RiPPs (bacteriocins) and NRPs. Antimicrobial, immunosuppressive, anticancer, and anti-inflammatory activities have been reported for at least some of these compounds, as well as potential therapeutic applications (58). The conventional clinically applied antibiotics have been sourced from BGCs, for example, erythromycin and tetracycline (polyketides), vancomycin (non-ribosomally produced peptides), neomycin (aminoglycosides), nisin (lantibiotic). clindamycin (lincosamides) (59) and others (Figure 4).

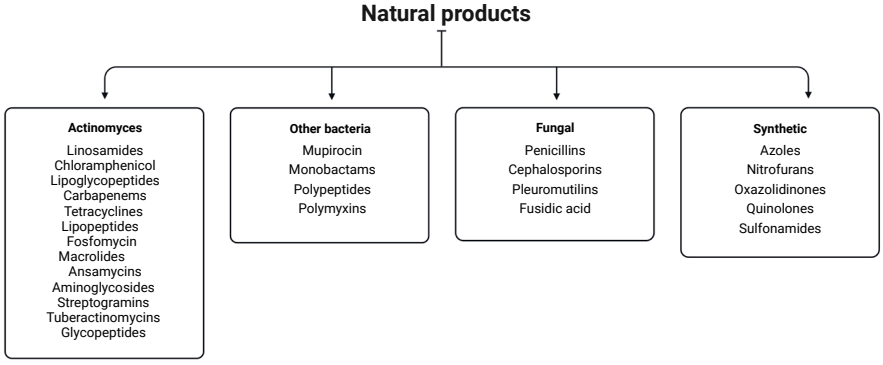

Figure 4. Clinically relevant classes of antibiotic that are derived from natural products. Adapted from (60).

Polyketides, are one of the largest classes of chemically diverse natural products with relevant applications in medicine, agriculture, and industry (58). They are assembled by polyketide synthases (PKS), multienzyme complexes that assemble a broad range of natural products including antibiotics (e. g. erythromycin and tetracycline) (61) and function by catalysing a repetitive condensation of acylco-enzyme A to form a polyketide chain (62). PKSs are classified into three types: type I PKSs that are structured in gene arrays with several functional domains found in bacteria, fungi and animals; type II PKSs that contain discrete catalytic domains that carry out iteratively acting enzymatic activities and are usually found in bacteria; and type III PKSs that include mainly chalcone synthase enzymes that catalyse product formation and occur mainly in plants and bacteria (63).

PK and NRP peptides are not chemically related but they have similarities with respect to their biosynthesis. Non-ribosomal peptides are natural products created by a family of assembly-line enzymes called non-ribosomal peptide synthetase (NRPS) (64), multi-modular enzymes that catalyse the biosynthesis of bioactive peptides that can be used for several therapeutic applications; well-known examples include the widely used antibiotics penicillin and vancomycin (65). Each NRPS module, generally includes a condensation (C) domain, an adenylation (A) domain, and a peptidyl carrier protein (PCP) domain. The respective modules select, activate and process individual amino acids into a growing peptide chain, forming the peptidyl backbone by sequential condensations (66). NRPSs are relatively small peptides (2–30 amino acids) with hallmarks of nonproteinogenic amino acids and extensive "post-translational modifications". Even though NRPS and PKS enzymes use different substrates, their conserved modular architecture, enzyme content and organization establish some compatibility which may result in hybrid NRPS-PKS assembly-lines that produce additional chemically diverse products (67).

RiPPs are produced from a precursor peptide which typically includes an N-terminal leader and a C-terminal core peptide isynthesized by ribosomes. The core precursor peptide is then biochemically modified and finally cleaved to generate the antimicrobial end product (68) (Figure 5). Multiple precursor types and sets of tailoring enzymes are responsible for the large structural diversity of RiPPs (69). RiPPs are classified by their modifications into different chemical families with conserved features and activities. Around 336 BGCs producing 41 classes and several unclassified RiPPs have been identified in bacteria, fungi, archaea, and plants (57, 68, 70). The three major classes of RiPPs are the lassopeptides, cyclotides and lanthipeptides (71). RiPPs often display narrow spectrum activity and have multiple mechanisms of action that can act simultaneously, reducing the likelihood of resistance development(71).

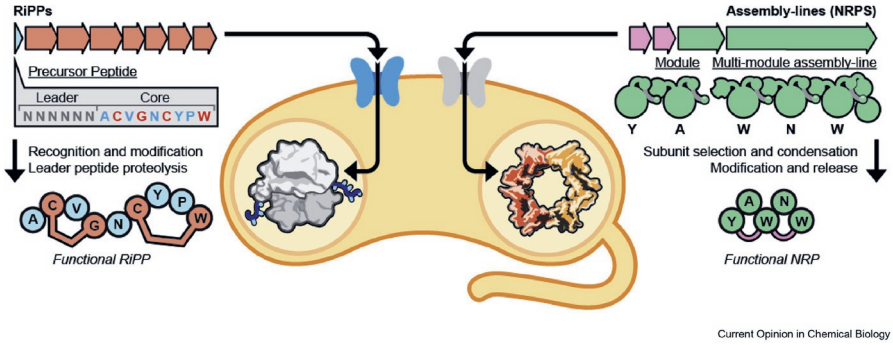

Figure 5. Structures and mechanisms of bacteria accessing RiPP and NRP antibiotic molecules. Figure adapted from (67) with permission from the Elsevier.

Overall, natural secondary metabolites are characterised by large biochemical and functional diversity and may mediate interactions that can influence evolutionary fitness of a microbial community through direct antagonism, niche defence, and signalling.

Conventional probiotics and next generation probiotics

Probiotics are defined by "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (72). Probiotic bacteria are able to reduce or inhibit of the growth of pathogens, for instance, by producing bacteriocins, siderophores, lysozymes, proteases, and hydrogen peroxides that are directly or indirectly correlated with positive effects (73). Probiotic modes of action also include the ability to regulate the host immune system and to adhere to the host epithelium (74). The application of probiotics as potential alternatives to antibiotics in human and veterinary medicine has been steadily rising over the years. In the past century, this approach led to the discovery of many conventional probiotic candidates, as *Saccharomyces boulardii* yeast, and lactic acid bacteria such as *Lactobacillus* and *Bifidobacterium* species, that have been commonly used as dietary supplements and additions to functional foods (75). For instance, the use of *Lactobacillus fermentum* and *Lactobacillus acidophilus* as additions to piglets feeds has shown increase in growth performances and decrease of *E. coli* counts and reduction of post-weaning diarrhoea (76). The addition of *L. salivarius* to animal feeds improved the immune status and reduced colonization by pathogenic bacteria in swine and also poultry (77). Consumption of *L. reuteri* by new born piglets brought beneficial effects on the expression of tight junction proteins which were suggested to reduce permeability of the maturing intestine (78). *E. faecium* was able to significantly mitigate incidence and severity of diarrhoea, leading to elevated daily weight gain in the piglets from birth to weaning (79). Probiotics based

on combinations of different species and strains have also been reported to decrease opportunistic infection, increase the growth performance of piglets and sows as well reduce overall mortality at weaning period (80, 81).

Abundant and prevalent species belonging to the core microbiota of healthy subjects are considered promising probiotic candidates to promote beneficial host-microbiota interactions (82). Extensive studies on microbiota interactions and therapies based on faecal microbiota transfer has stimulated interest in using commensals or symbionts as probiotics. These are commonly referred to as Next-Generation Probiotics (NGPs) and cover a broader range of genera and species than conventional probiotics (83). NGPs may also include recombinant micro-organisms developed for treatment of specific diseases under a medical regulatory framework (Table 1) (84, 85). Health-associated NGP candidates from the gut microbiota include strains from the genera *Bacteroides, Clostridium, Faecalibacterium* and *Akkermansia* (86). A consortium of 11 human bacterial species has shown

Table 1. Some characteristics of conventional probiotics and next-generation probiotics (NGP). Table adapted from (88).

Conventional probiotics	NGPs
• Isolated from gut, breast milk, and fermented foods.	• Mostly derived from commensals.
• Belong narrow range of organisms mainly *Lactobacillus* spp. and *Bifidobacterium* spp.	• Identified from comparison results between healthy and disease animals/people.
• Long history of safe use.	• Identified through research in microbiota-host interactions including NGS and multi-omics approaches
• Generally regarded as safe at the strain level by the US FDA or as qualified presumption of safety at the species level by EFSA.	• Belong to diverse genera including obligate gut anaerobes.
• Considered to be safe due to their food grade status (i.e. lactic acid bacteria used in food fermentation)	• Strict safety and regulations required.
• Do not include genetically modified microbes and are not marketed with claims for disease treatment.	• Include genetically modified bacteria for therapy of a specific disease
• Other genera include *Streptococcus* spp., Bacillus spp., *E. coli*, and *S. cerevisiae* (yeast), etc.	• Extra safety tests and mechanisms information required

EFSA: European Food Safety Authority; NGS: Next-Generation Sequencing; US FDA: USA Food and Drug Administration

positive results reducing infection and promoting anti-cancer immunity by colonizing the cecal and colonic mucus (87).

Aims and outline of this thesis

Bacterial infectious disease is a major welfare problem in livestock farming and in the absence of cross-protective vaccines antibiotics are still heavily used in the industry. *Streptococcus suis* is the one of the main causes of mortality post-weaning piglet, despite the metaprophylatic use of antibiotics to control disease on affected farms. The main aims of the thesis was to increase our understanding of *S. suis* infection, and the potential role of the tonsillar microbiota in colonisation resistance to *S. suis.* A further goal was to translate our findings into a NGP based on members of tonsillar microbiota that promote development of a diverse microbiota in newborn piglets and reduce the risk of *S. suis* disease. The specific aims and approaches covered in each of the Chapters are outlined below (Figure 6).

The **first aim** of this thesis was to investigate how *S. suis* colonisation of the palatine tonsil may lead to systemic infection in piglets. We hypothesised that CD169+ macrophages are present in the tonsil and that due to their relatively weak killing activity, virulent *S. suis* strains may be able to survive or replicate within these cells in tonsillar lymphoid tissue. In **Chapter II** we demonstrate the presence of different subsets of myeloid cells in the lymphoid tissue of the porcine palatine tonsils and visualise bacteria crossing the epithelial lining of the crypts and presence of bacteria in the tonsillar lymphoid tissue. We confirmed that large numbers of CD169+ macrophages are present in the tonsillar lymphoid tissue and also lining the tonsil crypts.

The **second aim** was to characterise health-associated tonsil microbiota and to generate a collection of tonsillar bacterial isolates representing different species, with the ultimate goal of identifying new antimicrobials. In **Chapter III** we describe the composition of the tonsillar microbiota of over 100 samples obtained from tonsil swabs taken from healthy piglets. 518 cultured isolates, representing 60 species from 23 genera, were screened for ability to inhibit growth of *S. suis* and other commensals and pathogens species from the tonsil microbiota. The culture-dependent approach was combined with genomic sequencing and use of antiSMASH software to identify novel biosynthetic gene clusters (BGCs) which might produce natural products including antimicrobials.

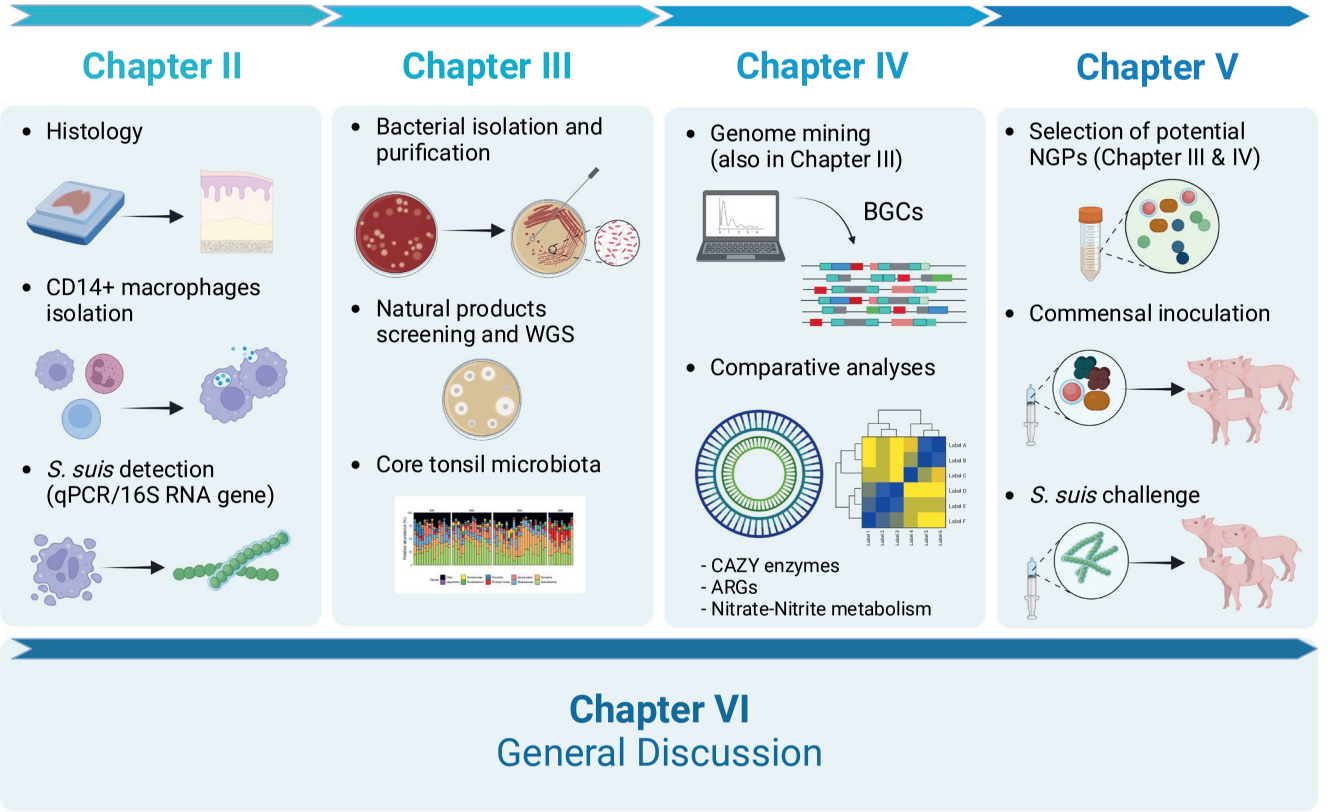

Figure 6. Schematic overview of the chapters of this thesis.

The **third aim** was to identify and characterize the genomes of a collection of Rothia species and strains which produce natural antibiotics. **Chapter IV** presents a detailed characterization of members of the genus *Rothia* which includes antibiotic producing species inhibiting *S. suis*. We identified BGCs predicted to produce antibiotic non-ribosomal peptides, iron scavenging siderophores and other secondary metabolites that modulate microbe-microbe and potentially microbe-host interactions. Several competition mechanisms and metabolic specializations linked to ecological adaptation of *Rothia* species in different hosts and environments were revealed.

The **fourth aim** was to identify key abundant species present in the tonsil biofilms of healthy piglets and investigate their capacity to promote development of microbial communities that provide colonisation resistance against *S. suis* infection at weaning. **Chapter V** presents results of an *in vivo* study where piglets were colonised with different groups of candidate NGPs post-partum and then challenged 5 weeks later with virulent *S. suis*.

Finally, in **Chapter VI,** the main findings and outcomes of this research were discussed into a broader context, highlighting its contribution to our understanding of *S. suis* colonisation and invasion of the palatine tonsils as well as the composition and resilience of members of the tonsil microbiota. The culture collection of tonsil isolates including 2 newly proposed species provides a valuable resource for future research on new natural bacterial products and NGPs.

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Emmy Klieneberger

II

Macrophages and Monocytes in the lympho-epithelial tissues of porcine palatine tonsil: do they play a role in systemic infection of piglets with *S. suis*?

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To be submitted

Abstract

Streptococcus suis is a bacterial pathogen causing significant losses to the pork industry. Invasive disease is commonly associated with sepsis, meningitis, endocarditis, pneumonia, and arthritis. Subclinical carriage of pathogenic isolates on the tonsil is considered important in the epidemiology of infection especially in transmission within and between herds. Here we showed bacteria crossing the epithelial lining of the palatine tonsil crypts and the presence of bacteria in the lymphoid tissue. We found large numbers of CD169+ macrophages in the tonsillar lymphoid tissue and also lining the crypts. Live *S. suis* including pathogenic strains were isolated from isolated $CD14^+$ myeloid cells isolated from the tonsil. Based on these observations we propose a hypothetical model to explain systemic infection of piglets with *S. suis*. Moreover, we suggest that virulent strains may be able to survive or replicate within these macrophages as shown for *Streptococcus pneumoniae* in CD169+ macrophages in the liver.

Keywords: *Streptococcus suis*; CD169; palatine tonsil; invasion; lymphoid tissue

Introduction

Streptococcus suis is a bacterial pathogen causing significant animal welfare issues and economic losses to the swine industry. Young pigs (weeks 4 to 12) are most at risk from invasive disease (1), with symptoms such as acute sepsis, meningitis, endocarditis, pneumonia and arthritis often being reported (2, 3). *S. suis* is also an emerging zoonotic pathogen of humans with highest incidence in Southeast Asia (4).

A recent longitudinal study on the tonsillar microbiota development in piglets showed *S. suis* colonisation of the palatine tonsil surface occurs rapidly after birth, reaching a relative abundance of around 10% (5). Streptococci and *S. suis* are prominent members of the tonsil associated microbiota, independently of *S. suis* disease (6, 7). The tonsil epithelium colonized by a combination of commensal non-disease-causing strains and pathogenic strains of *S. suis* (1, 8, 9). Subclinical carriage of pathogenic isolates is considered important in the transmission infection within and between herds (10). In experimental study, the inoculated *S. suis* strain was recovered 512 days after infection (11).

The palatine tonsils are considered the most important site of invasion by *S. suis* (12, 13). The tonsillar crypts extend deeply into the tonsil parenchyma and the stratified surface epithelium becomes single cell thick and lacks a basal lamina, enabling uptake or translocation of bacteria and particles from the oral cavity by or into the tonsils. After translocation, bacteria which avoid phagocytosis in the lymphoid tissue could travel through the efferent lymphatics to the bloodstream (14) or perhaps directly enter the circulation via blood vessels in the lymphoid tissue. The intestinal route of infection has also been investigated by inoculation or capsule-mediated delivery of virulent strains of *S. suis* into the small intestine of piglets (15). Considering the inoculum size, stress-inducing conditions, and small proportion of challenged animals developing sepsis, the translocation of *S. suis* across the intestine appears to be a plausible but not an efficient route of infection. The respiratory tract is a potential route of entry into the body for virulent strains of *S. suis*, either as single causal organism or in the context of co-infections with porcine respiratory viruses or polymicrobial infections (7, 16). Both routes and modes of infection lead to challenges of the immune system. A previous study using triple colour immunofluorescence histology showed that after infection of gnotobiotic piglets *S. suis* was associated with CD172-positive myeloid cells in the lymphoid tissue of the porcine palatine tonsils (12). CD169+ macrophages have not yet been identified in porcine tonsil lymphoid tissue but in rodents, CD169+ macrophages are known to be important in promoting adaptive immune responses to pathogens (17). Pathogens entering lymphoid tissue are first encountered by CD169+ macrophages which retain a source of antigen for cross-presentation to T

cells by dendritic cells (DCs) (18). Activated CD169+ macrophages also produce type I interferons to induce DC maturation and promote T cell activation (19).

Recent evidence that *S. pneumoniae* can proliferate in splenic CD169+ macrophages raise the possibility that virulent *S. suis* might escape killing and replicate in porcine CD169+ cells in the tonsillar lymphoid tissue. After release from infected macrophages, virulent *S. suis* bacteria might enter the lymphatics and blood vessels to cause sepsis (20). CD169 is a sialic acid binding immunoglobulin-like agglutinin (Siglec)-1 which may enhance phagocytosis of *S. suis* serotypes containing sialic acid in their capsule polysaccharide, which includes those serotypes, strongly associated with pathogenesis (9).

This study shows that bacteria are present deep within the crypts of the pig palatine tonsils and in close contact or inside lymphoid tissue cells lining the crypt. Different subsets of macrophages are present in the tissue, the most abundant being CD169+ macrophages which are associated with the crypts and areas around the germinal centres, supporting their proposed 'gatekeeper' role. (18). Viable *S. suis,* including pathogenic strains, were recovered from monocytes and macrophages isolated from the tonsillar lymphoid compartment suggesting that CD169+ macro- phages might permit sufficient intra-cellular survival of *S. suis* to allow its escape through the efferent lymphatics into the bloodstream.

Materials and Methods

Tonsil collection and microbiota sampling

The tonsil lymphoid organ from two healthy piglets of 9 weeks of age, sampled at farm Van Beek SPF pigs BV (Lelystad) were transported in 30mL of tissue culture medium (RPMI1640) with 10% Fetal Bovine Serum (FBS) and antibiotics (15ug/mL of vancomycin plus 50ug/mL of amoxicillin) to kill bacteria on the tonsil surface. Prior to dissection, tonsillar biofilm swabs were collected to define microbiota composition by 16S rRNA gene sequencing (see below) by brushing both palatine tonsils thoroughly with a sterile Puritan HydraFlock Swab placed directly into 2 mL vials containing garnet beads with 1mL of PowerBead Solution (Qiagen).

Histology

To visualize the tonsil crypts and lymphoid structures by microscopy, palatine tonsil tissue was fixed in 4% PFA (paraformaldehyde), then embedded in paraffin. Sections (5mm-thick) were cut with a microtome and stained with H&E and Gram-stain. For immunofluorescent detection of myeloid cell types, the tonsil tissue was frozen in liquid nitrogen, cut on a cryostat (5 µm thick sections), airdried for 30 min, fixed

in cold acetone for 15 min, and then added to cold TBS buffer. Immunohistochemistry was performed with the following antibodies; primary antibodies: CD169 (MCA2316GA, Biorad), CD163 (MCA2311-FITC Biorad), MIL11 (Prof. M. Bailey, Department of Clinical Veterinary Science, University of Bristol) CD16 (MCA1971F, Biorad); secondary antibodies: GAM-Alexa 488 (A11001, Invitrogen), GAM-alexa555 (A21422, Invitrogen), IgE-biotin (1110-08, Southern-Biotech). The secondary biotinylated antibodies were detected with Streptavidin-546 (S11225, Invitrogen). In between incubations with the antibodies, sections were rinsed with TBS and finally counterstained with Hoechst (33258) and embedded in Fluoromount-G (0100-01 Southern-Biotech).

Isolation of CD14+ cells from tonsils and bacterial DNA extraction

To generate a single cell suspension of the lymphoid tissue, mechanical dissociation was carried out by first excising the top layer of epithelial tissue and then cutting, chopping and scraping the internal tissue into small pieces. The dissociated tissue was then rinsed with sterile Phosphate-buffered saline (PBS) followed by gentle agitation in order to dissociate individual cells. Dissociated lymphoid cells were pelleted, resuspended in cold RPMI1640 containing 10% FBS and antibiotics (15µg/mL of vancomycin plus 50µg/ml of amoxicillin) and passed through a 40 µm single cell filter and collected on ice. The cells were then incubated with magnetic CD14 capture MicroBeads (Miltenyi Biotec) and isolated using a magnetic separation (MS) column (Miltenyi Biotec), according to manufacturer's recommendations (Figure 1). Cell counting was performed using an automated cell counter (Invitrogen). The positively selected CD14 (CD14+) cells were then incubated one hour with antibiotics to kill any extracellular bacteria. The purified CD14+ cells were then osmotically lysed by incubation in sterile demi water for 5-15 min and aliquots were plated as described below. The extraction of DNA from the suspension of CD14+ cell lysates containing the internalized bacteria were performed using PowerSoil Genomic Purification Kit (Qiagen), according to the manufacturer's recommended protocol.

Bacterial identification and DNA isolation

To culture viable bacteria associated with CD14+ cells, 100µL aliquots of the lysed cell suspension were plated on Brain Heart Infusion (BHI) agar. After overnight incubation at 37 ºC in presence of 5% CO_2, bacteria were counted and selected based on morphology for qPCR identification. Genomic DNA from the swab was extracted using the PowerSoil Genomic Purification Kit (Qiagen), according to the manufacturer's recommended protocol. *S. suis* was quantified by qPCR using primers specific for the V3-V4 region of the *S. suis* 16S rRNA gene; pathogenic isolates were quantified using primers targeting a conserved marker gene VM1, found only in pathogenic strains (unpublished data from UCAM and WUR).

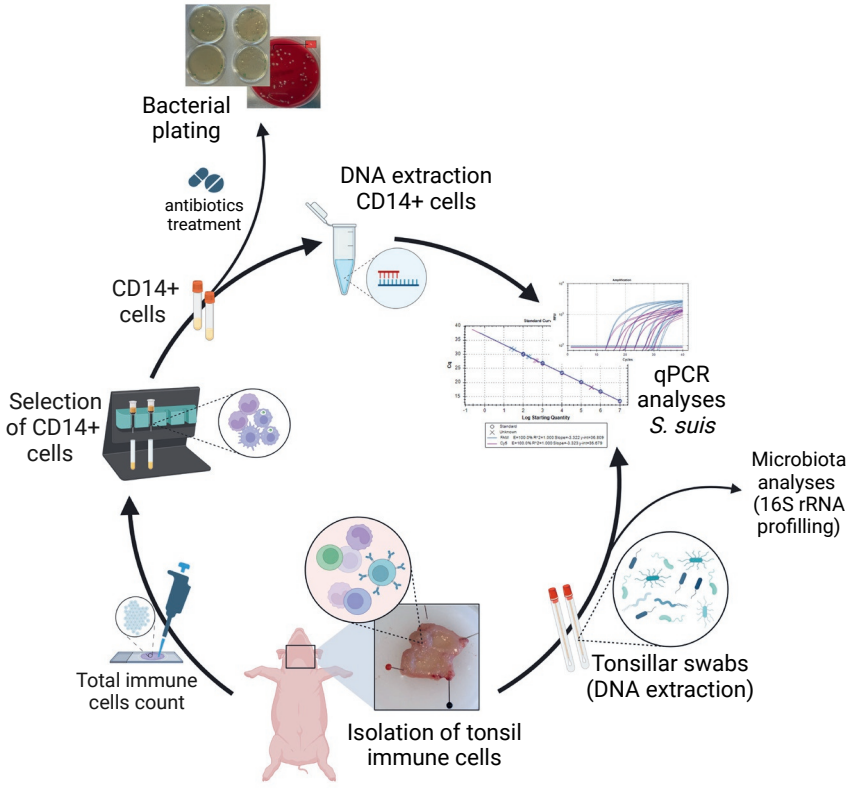

Figure 1. Schematic overview of the workflow to obtain bacteria associated with CD14+ myeloid cells. DNA isolation of tonsil swabs and tonsillar tissue was used to isolate, identify and quantify the presence of *S. suis* associated with CD14+ cells followed by culture of viable bacteria and qPCR analyses. Illustration generated using BioRender (https://app.biorender.com).

Results

Histology of tonsillar sections

The architecture of the porcine palatine tonsils consists of a stratified epithelium which forms deep crypts where the epithelial cell layer becomes one cell thick (Figure 2A). Bacteria are typically seen within the crypts but also within the lymphoid areas (Figure 2B, C). CD169+ macrophages are the most abundant in the lymphoid tissue, populating the tonsil crypts and surrounding the B cell follicles where T cells are also present (Figure 3C). This closely resembles the localization of CD169+ macrophages in the porcine and mouse spleen (not shown) (20). (20). The CD163

protein is a marker of most macrophage subpopulations (21) and potentially extravasated monocytes which are less abundant than CD169+ macrophages and not surrounding the B cell germinal centres.

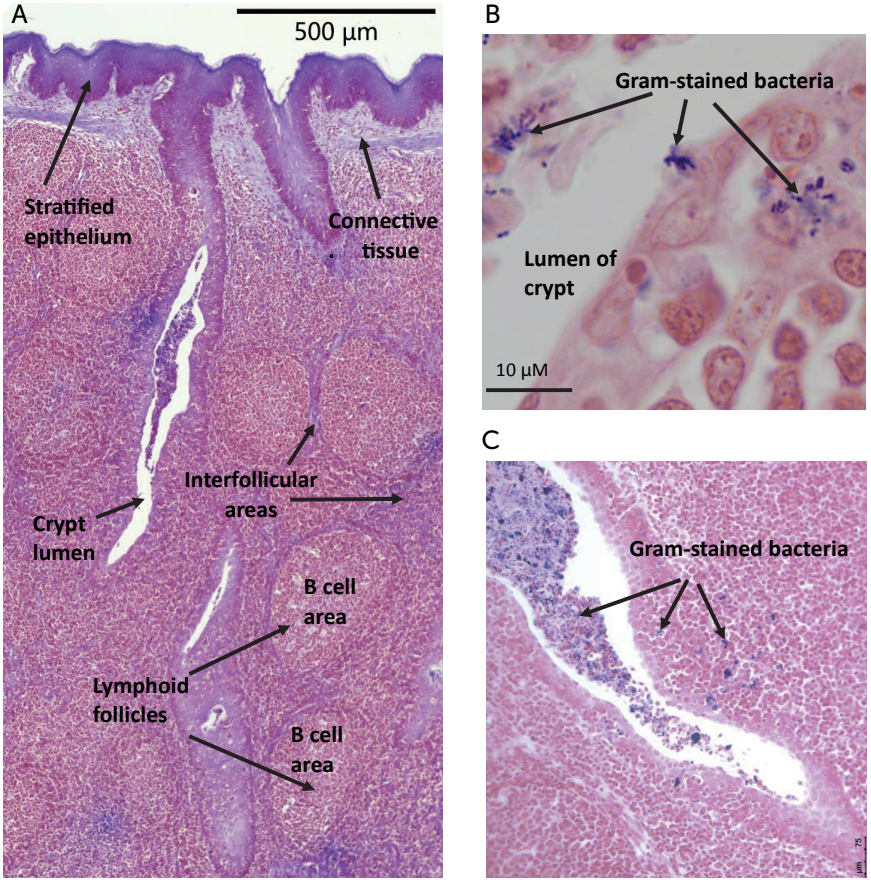

Figure 2. Fixed histological sections of porcine palatine tonsils stained with Gram-stain. **(A)**. Gram-positive bacteria are seen in contact with macrophages lining the crypt **(B)**. Bacteria can be seen deep in the crypts **(C)**.

Microbiota and *S. suis* associated with the tonsil epithelium

Prior to removal of the tonsil epithelium and resection of the lymphoid tissue we swabbed the surface of the tonsil for DNA extraction, bacterial 16S rRNA sequencing and qPCR to enumerate total and virulent *S. suis*. 16S rRNA gene profiling showed that the genera *Streptococcus, Actinobacillus, Moraxella* and *Rothia* were the most

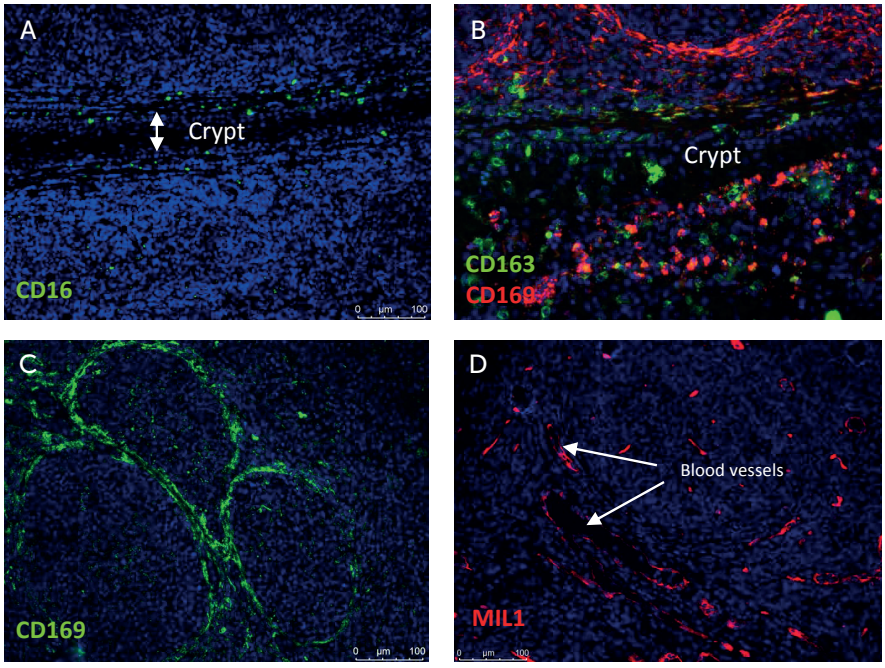

Figure 3. Immunofluorescence staining for the identification of specific markers in the tonsil lymphoid tissue. **(A)** CD16-positive dendritic cells (green) and cell nuclei (blue). **(B)** CD163-positive cells (green) and CD169-positive subset of macrophages (red), cell nuclei blue. **(C)** CD169-positive subset of macrophages (green), and cell nuclei blue. **(D)**. MIL1-positive endothelial cells lining the blood vessels (red) and cell nuclei (blue).

abundant genera in the swab samples collected in this study (Figure 4A). The relative abundance of the genus *Rothia* was greater in tonsil swab from animal 1 (SB1) than in tonsil swab from animal 2 (SB2); the relative abundance of the genus *Streptococcus* was higher in SB2. Within each genus, we detected a large number of rRNA amplicon sequence variants (ASVs), indicating the presence of multiple species and strains per genus. Several *S. suis* ASVs were detected, the most abundant of these being the 5th and 7th most common ASVs in sample SB1 and 1st and 16th most common in sample SB2 (Supplementary table 1).

Based on the qPCR in the DNA (2 uL of 1 mL) isolated from samples SB1 and SB2, we detected 2.25E+05 and 3.37E+05 target copies of the *S. suis* 16S rRNA gene, respectively. The total number of target copies per sample quantified by qPCR (Figure 4B) includes the 4 copies of 16S RNA gene copies per *S. suis* genome. There is only one copy of the virulence marker per genome of *S. suis*.

To quantify the target copies of pathogenic strains of *S. suis* strains we performed qPCR with primers targeting virulence marker (VM1) (see Methods). The target copy number of VM1 was 3.05E+01 for P1 and 3.63E+02 for P2 (Table 1). This confirms a previous study from our lab showing that pathogenic strains are present in substantially (up to a 1000 times) lower amounts in the tonsil microbiota than the commensal strains (Figure 4B) (unpublished).

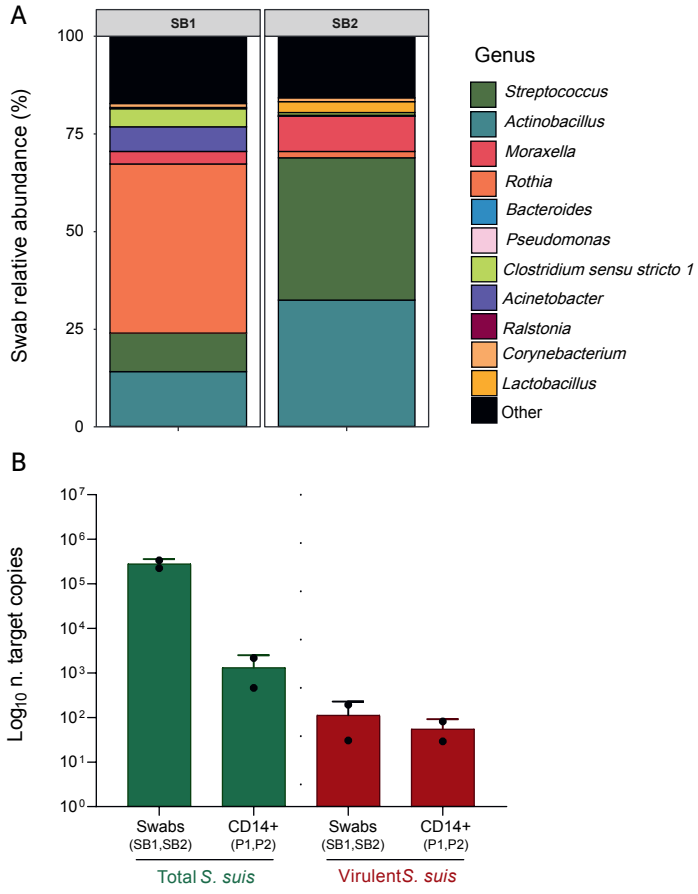

Figure 4. Microbiota composition and *S. suis* associated with the tonsil epithelium. **(A)** Tonsillar microbiota composition prior to dissection of the swab tonsil biofilm in the piglet one (SB1) and the swab of the piglet two (SB2). **(B)** Quantification of total amount of *S. suis* in tonsil swab and in the lysed CD14+ cells from piglet one (P1) and piglet two (P2). Green bars display quantification of the total amount of *S. suis* based on target copies of 16S rRNA gene, red bars display counts of virulent strains of *S. suis* based on single-target copies of virulence marker VM1.

Recovery and identification of bacteria associated with CD14+ macrophages and monocytes

From the lymphoid tissue sample of P1, approximately 7.32E+07 cells were obtained of which 1.37E+07 were isolated by immunomagnetic separation using anti-CD14 beads. After cell lysis and plating onto BHI agar we recovered 1.26E+05 CFU of viable bacteria from the plated suspension of 1.37E+07 purified macrophages and monocytes. From the lymphoid tissue of P2, 6.96E+07 cells were obtained of which 4.10 E+06 were isolated by anti-CD14 beads. After cell lysis and plating 1.13E+05 CFU of viable bacteria were recovered from the purified suspension of CD14+ macrophages and monocytes (4.10E+06) from P2 (Table 1). The target copies of *S. suis* in CD14+ cell lysate was 2.16E+03 for piglet one (P1) and 4.62E+02 for piglet two (P2) (Figure 4B).

To determine whether live *S. suis* could be recovered from the isolated CD14+ cells we picked 30 bacterial colonies with a colony morphology and size resembling *S. suis* for identification by qPCR. Seven of the 30 colonies were identified as *S. suis* and three of these were also positive for VM1. These results show that live *S. suis* and other bacterial species can be recovered from immuno-magnetic separated tonsil macrophages.

Table 1. Quantification of bacteria and *S. suis* associated with the macrophage cells isolated from the palatine tonsils.

				S. suis qPCR (target copies*)	
		Nº of immune cells	Bacteria (total CFU)	16s rRNA	VM1
	Tonsil swab SB1	-	-	2.25E+05	3.05E+01
P1	Total cell suspension	2.43E+07	-	-	-
	CD14 +	1.37E+07	1.26E+05	2.16E+03	8.12E+01
	Tonsil swab SB2	-	-	3.37E+05	3.63E+02
P2	Total cell suspension	6.96E+07	-	-	-
	CD14 +	4.10E+06	1.13E+05	4.62E+02	2.91E+01

P1; piglet 1, P2; piglet 2, SB1; swab from piglet 1, SB2; swab from piglet 2. **S. suis* qPCR raw counts, nor corrected for multiple (average of 4) target copies of the rRNA gene per genome.

Discussion

We identified *S. suis* on the surface of the palatine tonsil in relatively high abundance corroborating previous studies (8, 22). Our histological detection of bacteria in the deep crypts of the tonsil epithelium and in the lymphoid compartment is also consistent with other studies suggesting that bacteria and particulate antigens from the tonsil biofilm and saliva are found in the crypts and can enter the lymphoid compartment (12, 13).

Different subsets of macrophages as well as dendritic cells were identified in the lymphoid compartment of which the CD169+ macrophage subset were most abundant. CD169+ macrophages were located near the crypt boundary and around the follicular germinal centres. This is consistent with their proposed function as gatekeepers that phagocytose antigens, bacteria and viruses entering the lymphoid tissue and function in concert with dendritic cells to activate T cell responses (23). In the case of systemic infection caused by *Streptococcus pneumoniae*, it was shown that after capture by CD169+ macrophages in the spleen, *S. pneumoniae* replicates intracellularly leading to cell lysis and dissemination of the infection (24). This may be a consequence of the weak killing activity of CD169+ macrophages which seem to be mainly adapted for antigen capture and presentation to B and T cells (18, 25).

In conclusion we have shown that live *S. suis* can be recovered from the lymphoid tissue of tonsillar macrophages and present a working hypothesis to explain how virulent *S. suis* may survive in the tonsil, enter the efferent lymphatics and ultimately, the bloodstream, to cause sepsis. We show that CD169+ macrophages are abundant in the porcine tonsil lymphoid tissue. CD169 is a Siglec 1 receptor for sialic acid rich polysaccharides which may explain why *S. suis* serotypes containing sialic acid in their capsule polysaccharide are more commonly associated with invasive disease.

Data availability statement

All data generated for this study are included in the manuscript and/or in the Supplementary Files and at NCBI under the BioProject PRJNA926952.

Ethics statement

This study used samples obtained for diagnostic procedures performed according to the ethical principles and guidelines covered by EU Directive 2010/63/EU.

Funding

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Supplementary Table 1: Relative abundance of the 20 most abundant ASVs from SB1 and SB2.

	Swab sample one (**SB1**)			Swab sample two (**SB2**)		
	ASV	**Relative abundance**	**species**	**ASV**	**Relative abundance**	**species**
1	ASV00002	43.13	*Rothia_endophytica/mucilaginosa/nasimurium*	ASV00003	19.96	*Streptococcus_suis*
2	ASV00007	6.79	*Actinobacillus*_Unclassified	ASV00007	19.13	*Actinobacillus*_Unclassified
3	ASV00025	4.95	Unclassified_Unclassified	ASV00004	9.60	*Streptococcus_porcorum*
4	ASV00010	4.47	*Clostridium sensu stricto 1*_Unclassified	ASV00005	8.15	*Actinobacillus_minor*
5	ASV00011	4.40	*Streptococcus_equinus/suis*	ASV00008	4.23	*Moraxella_porci*
6	ASV00015	3.56	*Acinetobacter_gandensis*	ASV00025	3.72	Unclassified_Unclassified
7	ASV00003	2.99	*Streptococcus_suis*	ASV00026	3.26	*Streptococcus*_Unclassified
8	ASV00012	2.26	*Moraxella_pluranimalium*	ASV00030	2.86	*Moraxella*_Unclassified
9	ASV00022	2.05	*Actinobacillus_porcinus*	ASV00024	2.84	*Actinobacillus_A. indolicus/H. parasuis*
10	ASV00005	1.91	*Actinobacillus_minor*	ASV00012	1.92	*Moraxella_pluranimalium*
11	ASV00019	1.52	*Actinobacillus_minor/porcitonsillarum*	ASV00027	1.88	*Conchiformibius*_Unclassified
12	ASV00028	1.51	*Actinobacillus_arthritidis/equuli/lignieresii*	ASV00031	1.44	*Fusobacterium_necrophorum*
13	ASV00032	1.40	*Terrisporobacter*_Unclassified	ASV00036	1.16	*Lactobacillus_acidophilus/amylovorus/ultunensis*
14	ASV00004	1.38	*Streptococcus_porcorum*	ASV00019	1.04	*Actinobacillus_minor/porcitonsillarum*
15	ASV00054	1.22	*Acinetobacter*_Unclassified	ASV00058	0.93	*Corynebacterium*_Unclassified
16	ASV00038	0.90	*Acinetobacter_defluvii*	ASV00035	0.92	*Streptococcus_henryi/orisratti/porcorum/suis*
17	ASV00047	0.77	*Corynebacterium_glutamicum*	ASV00033	0.88	*Streptococcus_equinus/gallolyticus/macedonicus*
18	ASV00008	0.66	*Moraxella_porci*	ASV00040	0.83	*Actinobacillus_indolicus*
19	ASV00052	0.61	*Chryseobacterium_culicis/indologenes/jejuense*	ASV00094	0.80	*Porphyromonas*_Unclassified
20	ASV00066	0.42	*Aerosphaera*_Unclassified	ASV00043	0.73	*Rothia*_Unclassified


Anno XIV
GAZETA
BRAZIL
Streptococcus suis is a Gram-positive bacterium which causes
meningitis, sepsis, and other diseases in swine. Disease-associated
S. suis strains are
espiratory disease
in piglets.
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Dr. Ruth Ella Moore

III

Identification of novel species and biosynthetic gene clusters producing antimicrobial molecules through culturomics of the porcine tonsil microbiota

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Submitted for publication

Abstract

Background: In humans and pigs altered composition of the microbiota associated with the epithelium of the palatine tonsils has been associated with bacterial or viral infection and lymphoid tissue inflammation. The tonsil lymphoid tissue is important for immunity and considered the main portal of entry for pathogenic *Streptococcus suis*. Little is known about correlations between tonsil associated microbiota, tonsillar infections and the species which might confer colonisation resistance against pathogens. Here we established a large collection of representative bacterial species from the tonsil surface biofim and used genome mining and *in vitro* assays to assess their potential as probiotics to reduce infections with *S. suis* and other pathogens.

Results: : Data on tonsil microbiota composition from over 100 piglets from 11 farms and three countries revealed a core microbiota comprising *Actinobacillus, Streptococcus* and *Moraxella* and 11 other less abundant but prevalent genera. To establish a collection of culturable core species we plated 5 tonsil swabs taken from healthy piglets on different farms and countries on different media and isolated 518 pure cultures belonging to 23 genera. To identify candidate probiotic strains, we employed pathogen inhibition screenings and *in silico* genome mining to assess (i) antagonism toward a panel of disease-associated bacteria, and (ii) presence of biosynthetic gene clusters (BGCs) in genomes of isolates that might produce antimicrobial compounds. We characterized two novel species: one *Brevibacterium* species and one *Corynebacterium* species which produced a new variant of the lanthipeptide Flavucin that inhibits *in vitro* growth of opportunistic pathogens as *S. suis* and *S. aureus*.

Conclusions: We defined the core tonsil microbiota of piglets and cultured representative single bacterial isolates for future research on tonsil microbiota–host interactions in the oral cavity. Several isolates showed potential to inhibit growth of common bacterial pathogens and might be exploited as probiotics to promote the development of a healthy tonsil microbiota in young piglets and provide colonisation resistance against pathobionts or obligate pathogens. The mining of genomes from cultured isolates suggests that the tonsil microbiota is an untapped source of novel antimicrobials.

Keywords: culturomics • porcine palatine tonsil • microbiota • biosynthetic gene cluster • upper respiratory tract

Introduction

Culturomics is commonly defined as a culturing approach to obtain a diverse collection of bacterial isolates that may represent novel species and strains variants, using multiple culture conditions. Matrix assisted laser desorption/ionisation - time of flight (MALDI-TOF) mass spectrometry and 16S rRNA gene sequencing are commonly used to identify the taxonomy of isolates obtained by repeated selection of single colonies on agar plates. Functional screening and genome sequencing of isolate collections from environmental and host-associated microbial communities is important for future research on host-microbe interactions and the discovery of novel species and biosynthetic gene clusters (BGCs) producing bioactive molecules that mediate microbe-microbe and microbe-host interactions (1).

Once characterized and archived as a pure culture, strains with symbiotic or beneficial activities can be used for translational research on host-microbe interactions or for biotechnological applications. Identification and (co-)culture of niche-specific microorganisms is an important strategy to gain knowledge on community structure and antagonism between symbionts and pathobionts. One example is *Staphylococcus lugdunensis* which produces lugdunin, a thiazolidine-containing cyclic peptide antibiotic that inhibits colonisation by *Staphylococcus aureus* in the nasal cavity. Human nasal colonization by *S. lugdunensis* was associated with a significantly reduced carriage of *S. aureus* (2, 3). The production of natural antimicrobials is an adaptative advantage for bacteria and drives ecological processes such as colonization and niche persistence (4, 5). A previous study of human microbiomes in several body sites revealed specific bacteria harboring BGCs, which led to the discovery of a novel thiopeptide lactocillin that was active against multiple Gram-positive pathogens (6). Other small molecules produced by the microbiota influence bacterial growth, motility, nutrient acquisition, stress response and biofilm formation (7).

The palatine tonsils, located on the ventral side of the soft palate, are immune tissues where microorganisms from the mucosal surface or oropharyngeal fluids that enter the epithelial crypts are sampled by macrophages and dendritic cells to elicit mucosal immune responses that protect the respiratory tract (8, 9). Despite the key role tonsils play in innate, cellular, and humoral immunity, the tonsil-associated microbiota has been less studied than the dental, periodontal and nasal microbiota (8, 10).

The tonsil surface is the main host niche for *Streptococcus suis*, a bacterial species that contains commensal strains but also disease-associated (or virulent) strains

that may cause meningitis and sepsis in piglets in the first 2-4 weeks post-weaning (11). Asymptomatic carriage of pathogenic strains of *S. suis* in piglets is common on farms with a recent history of *S. suis* disease. Reduced host immunity, co-infections, and stress also appear to contribute to risk of *S. suis* disease (11). Recent studies have shown that the abundance of several tonsil colonizing species is associated with *S. suis* disease, and that reduced microbiota diversity pre-weaning may predispose to *S. suis* disease post-weaning (12, 13). Oral administration of isolates that antagonise adherence or growth of *S. suis* may help to reduce *S. suis* carriage and risk for invasive disease as shown for colonization by *S. lugdunensis* and nasal carriage of *S. aureus* (2).

The aim of this study was (i) to determine the core microbiome of healthy piglets on European farms without a recent history of *S. suis* cases, and (ii) perform culturomics using the swab samples from the same farms to establish a collection of strains part of the health-associated core microbiota. We cultured more than 500 isolates representing abundant and prevalent bacterial species. The collected cultivable tonsillar bacteria were screened for their capacity to produce natural products antagonising *S. suis* and other disease-associated or commensal bacteria. In total 45 genomes were sequenced and the genome mined for the presence of gene clusters predicted to produce secondary metabolites with potential anti-microbial function. This study identified widespread distribution of different classes of small-molecule-encoding BGCs and contribute to our knowledge of the tonsil microbiota of piglets.

Material and Methods

1. Animals and swab samples

101 samples were obtained from 101 piglets collected on fifteen farms in three countries: Germany, The Netherlands and Spain (Table 1). The 101 healthy piglets were randomly selected one week before weaning (3 weeks-old; timepoint -1) and three weeks after weaning (timepoint +3) and tonsillar biofilms were collected from the surface of the palatine tonsil using Puritan HydraFlock Swabs (Daklapack, Europe).. Samples were collected from piglets between 3 and 8 weeks old. After sampling, swabs were kept on ice and upon reaching the lab, were stored at -80 °C until further processing. Samples from five animals from three farms (ES1, ES2 and NL1) were used for culturomics. Swabs used for culturomics were transferred to a tube containing 2 mL sterile of buffered peptone water (BPW), a non-selective pre-enrichment medium, containing 15% glycerol used to preserve bacterial viability. Samples were stored at -80°C within 4 hours of collection. Swabs used for

DNA extraction and microbiota analyses were placed directly after sampling into 2 ml vials containing garnet beads with 1mL of PowerBead Solution (Qiagen).

2. 16S rRNA gene amplicon sequencing of microbiota samples

The V3-V4 region of 16S rRNA gene was amplified with primers 341F (CCTAYGGGR-BGCASCAG) and 806R (GGACTACNNGGGTATCTAAT) and 250 bp paired end sequencing with Illumina HiSeq 2500 or Novaseq 6000 sequencing platforms. Reads were trimmed with cutadapt 2.3 (14) using default settings before being processed in DADA2 (15) v1.4 workflow for paired end data. Taxonomy was assigned with SILVA database v132 (16). Amplicon sequence variants (ASVs) with taxonomic assignment as eukaryote or chloroplast were discarded, and read counts were rarefied to the minimum library size (51,577 reads). Alpha and beta diversity were calculated using R packages phyloseq (17) and vegan (18) and the adonis function in vegan was used to perform permutational analysis of variance (PERMANOVA) test; this multivariate ANOVA calculates strength and statistical significance of sample groupings based on a dissimilarity matrix. The criteria used to define the core microbiota were: minimum abundance of 0.1% per sample, and at least 90% prevalence over the 101 samples.

3. Isolation and culture conditions

For culturomics, swabs in transport medium and 15% glycerol were taken from the -80 °C storage and for each sample, dilutions between 10^{-3} and 10^{-6} were plated onto eight different culture media: Sheep blood agar (SBA) (Becton Dickinson, Heidelberg/Germany), Brain Heart Infusion (BHI) (Becton Dickinson, Heidelberg/Germany), M17 (Sigma-Aldrich/Germany), de Man, Rogosa and Sharpe (MRS) (VWR International, Leuven/Belgium), Mueller Hinton (MH) (Oxoid Ltd, Basingstoke), Todd-Hewitt Broth (THB) (Oxoid, Basingstoke/UK), Luria-Bertani (LB) medium (Merck, Darmstadt/Germany) and Mac Conkey (MC) medium (Merck, Darmstadt/Germany). Single colonies were picked and transferred to the corresponding liquid medium. Pure cultures were grown on solid 1.5% w/v agar medium and incubated at 37 ◦ C under aerobic conditions with and without 5% CO_2, or at 37 ◦ C in an anaerobic chamber (Bactron, Sheldon Manufacturing) between 18 and 48 hours.

4. Bacterial identification

MALDI-TOF MS was used first for taxonomic identification of pure cultures. To this, samples were spotted in target grids and covered with 1µL of matrix solution (saturated α-cyano acid-4-hydroxycinnamic acid in 50% acetonitrile and 2.5% trifluoroacetic acid). An isolate was considered correctly identified at the species level when the colonies' spectra had score ≥1.9 and identified at the genus level when the spectra had score ≥1.7 (spectral data not shown) (19). Isolates for which

whole genome sequences were available had 16S RNA genes identified using Barrnap (version 0.9; https://github.com/tseemann/barrnap). When no genome sequence was available, taxonomic assignment was performed by sequencing near-full-length (>1100 bp) amplicons of the 16S rRNA gene obtained by colony PCR with the universal primers 27F (5'-AGAGTTTGATCMTGG-3') and 1492R (5'-ACGGGCGGTGTGTRC-3'). The following conditions were used for amplification using Bio-Rad thermocycler: one cycle of 98 ºC for 30 min, followed by 35 cycles of 98 ºC for 10 min, 60 ºC for 15 min, 72 ºC for 20 min, and one cycle of 72 ºC for 2 min. Prior to colony PCR, colonies were resuspended in 40µl of lysis buffer and 2 µL was used as a template to which were added 5 µL of Q5 reaction buffer (5 X), 0.5 µL of dNTPs (10mM), 1.25 µL of each primer (10µM), 0.25 µL Q5 Hot Start High-Fidelity DNA Polymerase (New England BioLabs Inc.) and Nuclease-Free Water to an end volume of 25 µL reaction mix. Expected sizes of PCR products were confirmed on 1.5% agarose gel using a 1Kb DNA Ladder (Invitrogen by Thermo Fisher Scientific). For each isolate, the corresponding 16S rDNA sequence was searched against 16S rDNA sequences provided by NCBI database by BLAST (20) and taxonomy was assigned using SILVA database v132 using criteria below. For phylogenetic analysis, multiple alignments of 16S rRNA gene sequences were generated using Muscle (21) and the corresponding phylogenetic trees were generated using Maximum Likelihood in FastTree v2.1 (22) (1,000 replicates). Consensus trees were visualized and annotated using the interactive Tree Of Life (iTOL) v5 (23).

5. Novel taxa

For initial delineation of novel species, we used BLAST similarity searches of full-length 16S rRNA genes at a threshold of 98.7% sequence identity. The Genome-to-Genome Distance Calculator 3.0 (GGDC) (http://ggdc.dsmz.de) was used to estimate genome-based delineation of species by digital DNA-DNA hybridization (dDDH), and FastANI (24) was used to calculate the Average Nucleotide Identity (ANI) with a threshold of 95% (25, 26). Species-level delineation was further confirmed by membrane fatty acid profiles (27). For two candidate novel species, under their respective optimal growth conditions, cellular fatty acid profiles were determined at the Leibniz Institute DSMZ. Approximately 300 mg (wet weight) of cell biomass was extracted according to the standard protocol of the Microbial Identification System (MIDI Inc., version 6.21). The composition of cellular fatty acids was identified by comparison with the TSBA6 6.21 library.

Brevibacterium moorei sp. nov.: *Brevibacterium moorei* was named in honor of the African-American Dr Ruth Ella Moore for her contribution to the field of bacteriology. *Corynebacterium kozikiae* sp. nov.: *Corynebacterium kozikiae* was named in honor

of the African-American microbiologist Dr Ariangela J. Kozik for her contribution to human respiratory microbiome research.

6. Inhibition assays and crude extraction of antimicrobial peptides

Pathogen inhibition screening (or antagonism) assays were performed using over-night (o/n) liquid cultures generated from pure bacterial colonies of the 518 cultured tonsil isolates. Five microliters of each o/n culture were spotted onto the appropriate agar medium to allow growth of bacterial colonies. Agar media with inoculated colonies were then overlaid with soft agar (0.75% w/v agar) that contained approximately 10E+05 CFU/mL of one of the following: disease-associated *S. suis* P1/7, *S. suis* S10 and its isogenic unencapsulated mutant J28, *Staphylococcus aureus* ATCC 6538P, *Pasteurella aerogenes* DMS 21448, *Moraxella pluranimalium* DSM 22804, *Escherichia coli* L4242, or a *ΔtolC* mutant derivative from *E. coli* MG1061 that does not produce the outer membrane channel protein TolC, a key component of multidrug efflux and type I secretion transporters (28). We also checked for inhibition of three streptococci that commonly occur in the microbiota of healthy piglets: *Streptococcus porci* strain DSM23759, *Streptococcus porcinus* strain DSM20725 and *Streptococcus parasuis* strain DSM29126. The antimicrobial activity of cultured strains was determined by the presence of clearly visible zones of growth inhibition around the colonies after overnight incubation.

To further characterize the activity identified in *C. kozikiae* strain 76QC2CO, glycerol stocks of isolate 76QC2CO were sub cultured in BHI media overnight at 37°C. From this overnight culture, BHI agar plates were inoculated and incubated for 48h at 37°C to obtain a lawn of growth. A volume of 5 mL sterile NaCl 0.9% solution was added and bacteria were detached from the agar surface by 5 min incubation at room temperature on a horizontal shaker followed by gentle scraping with a sterile plastic T-shaped spreader (VWR International, Amsterdam, The Netherlands). The cell suspension was collected in sterile 15 mL Falcon tubes and centrifuged for 15 min at 3200 x g at 4°C. Bacterial cell pellets were resuspended in 70% 2-propanol (IPA), 0.1% trifluoracetic acid (TFA) and stirred for 6 hours at 4°C. The IPA-TFA cell extract (CE) was obtained by centrifugation for 15 min at 10.000 x g at 4°C, and pressed through a 0.2 µm sterile syringe filter. To partially purify the antimicrobial peptide from the CE, the IPA-TFA fraction was removed by rotary evaporation, and the remaining sample used for agar diffusion assays, thermal sensitivity- and cytotoxicity assays. BHI agar (0.75% w/v agar) was cooled to 50°C and seeded with a selected target bacterium (approximately 1 x 10^6 CFU mL-1) (Table 3). A volume of 25 mL was transferred to sterile Petri dishes and after solidification, wells (4.6 mm diameter) were made using a disposable sterile Pasteur pipette (Corning®). A volume of 50 µL µL of the CE was dispensed into the wells and the plates were

incubated at 37°C overnight. Antimicrobial activity was determined by the presence and size of clearly visible zones of growth inhibition around the wells as described before (29).

7. Genome sequencing and genome mining for antimicrobial peptide-encoding genes

Whole genome sequencing (WGS) was carried out for 45 tonsillar isolates that were selected based on the presence of inhibition zones, and difference on the colony color, size and shape. Isolates that had shown antimicrobial activity against target bacteria were selected for WGS and genome mining. Prior to DNA extractions, strains were grown overnight in BHI broth at 37°C. Genomic DNA was extracted using the PowerSoil Genomic Purification Kit (Qiagen), according to the manufacturer's recommended protocol. Recovery of high molecular weight DNA was assessed on a 0.8% agarose gel (Sigma-Aldrich) in 1X TAE buffer [Tris-HCl 40 mM, 20 mM acetic acid and 1 mM EDTA (pH 8)]; DNA was stained with 25 µg/mL of SYBR Safe and quantified using the Qubit dsDNA Broad-Range (Invitrogen) assay and Invitrogen Qubit Fluorometer (Thermo Fisher Scientific).

We obtained full genome sequences for 45 tonsil isolates from five piglets, randomly selected from different litters on two Spanish farms with a high-health status. Genome sequencing was performed on an Illumina HiSeq 2000 platform (Illumina, Inc.) at MicrobesNG, Birmingham, United Kingdom. Reads were trimmed using Trimmomatic 0.30 software with a sliding window set at Q15 (30). Genome assembly was performed using SPAdes version 3.7 (31). All genomes were annotated using Prokka 1.14.6 (32). For identification and annotation of biosynthetic gene clusters (BGCs), draft genomes of the 45 bacterial strains were analysed using antiSMASH 6.0.1 (33) using default analysis settings. BAGEL4 was used to mine the genomes for RiPPs and their precursor peptides (34).

Results

16S rRNA gene diversity and core microbiota of healthy piglets

We analysed a total of 101 microbiota samples obtained as tonsil swabs of piglets from 11 farms located in Germany, The Netherlands, and Spain (Table 1) using 16S rRNA gene V3-V4 amplicon sequencing at a mean depth of 81, 028 reads (range: 51, 577-180, 000 after quality filtering and chimera removal; see Methods).

Table 1. Overview of the sampling distribution for the culturomics (ES1, ES2 and NL1) and microbiota analyses.

Country	Farm	Age	No of samples	Farm type
Germany	DE1	Postweaning	6	Commercial farming
Germany	DE2	Postweaning	6	Commercial farming
Germany	DE3	Postweaning	5	Commercial farming
Germany	DE4	Postweaning	4	Commercial farming
Germany	DE5	Postweaning	4	Commercial farming
Germany	DE6	Postweaning	4	Commercial farming
Spain	ES1	Postweaning	15	Commercial farming
Spain	ES2	Preweaning	16	Commercial farming
Spain	ES3	Preweaning	22	Commercial farming
Spain	ES4	Preweaning	10	Commercial farming
Netherlands	NL1	Postweaning	9	Research farm

DADA2 (15) identified 39312 unique 16S rRNA gene V3-V4 region amplicon sequence variants (ASVs) of which 33.06% did not have a matching entry in the SILVA v132 database (16) at species level across the 101 tonsil swab samples. The three most abundant genera present in the tonsil microbiota were *Actinobacillus, Moraxella,* and *Streptococcus*, adding up to 51.60% of mean relative abundance with 206 unique ASVs (Figure 1). At the genus level, after comparing relative abundance and presence/absence of taxa across samples, we propose that 14 genera with relative abundance greater than 0.1% and presence in ≥ 90% of the samples (before and after weaning) can be designated as core members of the tonsil microbiota. These core members comprised 80.36% of total taxonomic diversity of the piglet tonsillar microbiota (Figure 1).

At ASV level, the core piglet tonsillar microbiota included 17 different ASVs, two in the genus *Moraxella,* seven in the genus *Actinobacillus,* three in the genus *Streptococcus,* and one in the genera *Alloprevotella, Porphyromonas, Rothia, Prevotella* and *Fusobacterium* (Table 2).

To assess bacterial community composition across all samples and farms, Principal Component Analysis (PCA) on Bray-Curtis dissimilarity was conducted (Figure 2). The first two principal components explained 23.1% (14.5% and 8.6% for PC1 and PC2, respectively) of the total variance. We found significant differences between microbiota composition and observed that the samples clustered together primarily according to country of origin (PERMANOVA on Bray-Curtis dissimilarity, $p < 0.001$)

DE1	DE2	DE3	DE4	DE5	DE6	ES1	ES2	ES3	ES4	NL1	Mean	ASVs	Prevalence	
22.1	29.1	36.5	25.9	31.0	18.4	31.9	38.0	25.5	24.9	24.7	28.0	130	100	***Actinobacillus***
5.6	5.9	6.1	2.8	4.8	7.6	22.8	21.6	28.6	20.3	8.2	12.2	37	100	***Moraxella***
5.5	22.5	17.9	15.4	17.3	12.3	6.6	7.4	11.1	3.7	8.5	11.7	43	100	***Streptococcus***
3.1	2.8	0.7	3.2	3.2	5.9	1.8	2.7	2.7	17.6	8.3	4.7	42	100	***Porphyromonas***
2.2	2.9	1.1	7.4	3.2	7.8	4.2	3.9	3.3	6.5	3.0	4.1	17	100	***Alloprevotella***
9.0	3.9	5.0	2.8	2.5	2.2	4.6	2.4	1.8	1.6	2.2	3.4	45	100	***Prevotella***
2.7	1.2	0.4	6.4	2.4	2.4	1.7	2.4	2.3	4.5	2.7	2.7	13	100	***Fusobacterium***
2.2	1.2	0.7	4.2	3.0	1.5	1.6	1.5	2.6	1.6	4.7	2.2	11	94	***Acinetobacter***
3.8	3.8	4.1	3.8	3.7	1.7	0.6	0.9	0.6	0.1	0.9	2.2	13	92	***Lactobacillus***
1.4	0.8	0.6	1.5	1.4	5.8	0.5	1.2	1.0	2.1	3.2	1.8	22	100	***Bacteroides***
1.2	3.5	0.3	1.5	1.6	2.8	0.8	2.3	1.5	2.5	1.0	1.7	16	99	***Leptotrichia***
0.6	0.8	0.8	0.5	1.5	6.3	1.6	1.0	0.9	2.0	2.1	1.6	3	100	***Rothia***
0.4	3.9	1.1	3.4	1.7	3.7	0.5	0.5	0.4	0.2	0.5	1.5	11	85	*Veillonella*
1.9	1.4	0.3	0.9	1.1	2.0	0.6	0.6	0.9	1.7	0.9	1.1	9	100	***Neisseria***
1.3	0.7	1.6	0.8	0.6	0.5	2.3	0.8	0.6	0.0	0.9	0.9	6	83	*Clostridium*
0.8	0.3	0.1	1.0	0.6	0.7	0.1	0.1	0.3	0.3	4.1	0.8	4	67	*Alysiella*
0.1	0.1	0.2	0.3	0.3	0.4	0.6	1.2	3.5	1.0	0.1	0.7	8	70	*Bergeyella*
0.6	0.2	0.2	0.9	1.0	0.8	0.3	0.4	0.4	0.8	1.3	0.6	4	86	*Conchiformibius*
1.5	0.5	1.2	0.2	0.3	0.1	0.7	0.3	0.1	0.0	0.4	0.5	8	66	*Agathobacter*
1.7	0.6	1.6	0.3	0.4	0.1	0.3	0.1	0.0	0.0	0.3	0.5	5	54	*Subdoligranulum*

Figure 1. Heatmap displaying the most abundant and prevalent genera across all samples (n=101) collected in three countries in Europe (DE, Germany; NL, The Netherlands and ES, Spain). 14 core genera that occurred in the dataset after applying a 90% prevalence and 0.1% abundance threshold are displayed in bold font. The numbers in columns DE1 to NL1 represent the average relative abundance; ASVs = unique amplicon sequences variants per genus. Mean = overall average of the per-country averages.

as well as per farm. Samples from farms in Germany and Netherlands clustered more closely together compared to samples from Spanish farms in the PCA (Figure 2). PERMANOVA on Bray-Curtis dissimilarity also detected significantly different microbiota composition ($p < 0.001$) within each country. The Shannon alpha-diversity of the core genera microbiota varied slightly between countries (Supplementary Figure 1).

Table 2. ASV level core microbiota from 101 animals on 11 European farms from 3 countries collected in this study. An ASV was scored as part of the core microbiota in this study when it had been scored in ≥ 90% of the samples and ASV abundance in the respective microbiota sample had been higher than 0.1%.

ASV	Taxon	Family	Prevalence (%)	Mean Abundance (%)
ASV00006	*Streptococcus suis*	*Streptococcaceae*	100.00	2.06
ASV00018	*Streptococcus suis*	*Streptococcaceae*	99.28	1.44
ASV00002	*Moraxella pluranimalium*	*Moraxellaceae*	98.56	3.85
ASV00013	*Moraxella porci*	*Moraxellaceae*	98.56	1.72
ASV00020	*Porphyromonas* Unclassified	*Porphyromonadaceae*	98.56	1.30
ASV00003	*Alloprevotella* Unclassified	*Prevotellaceae*	97.12	2.73
ASV00027	*Actinobacillus indolicus/H. parasuis*	*Pasteurellaceae*	97.12	0.78
ASV00021	*Fusobacterium gastrosuis*	*Fusobacteriaceae*	96.40	1.06
ASV00029	*Prevotella* Unclassified	*Prevotellaceae*	94.96	0.60
ASV00007	*Actinobacillus porcinus*	*Pasteurellaceae*	93.53	2.43
ASV00015	*Actinobacillus minor/porcitonsillarum*	*Pasteurellaceae*	93.53	1.81
ASV00016	*Actinobacillus indolicus/minor*	*Pasteurellaceae*	92.81	1.38
ASV00008	*Actinobacillus indolicus/H. parasuis*	*Pasteurellaceae*	92.09	1.42
ASV00023	*Actinobacillus minor/indolicus*	*Pasteurellaceae*	92.09	0.69
ASV00009	*Actinobacillus porcinus*	*Pasteurellaceae*	91.37	2.17
ASV00036	*Rothia nasimurium*	*Micrococcaceae*	91.37	0.53
ASV00019	*Streptococcus suis*	*Streptococcaceae*	90.65	1.32

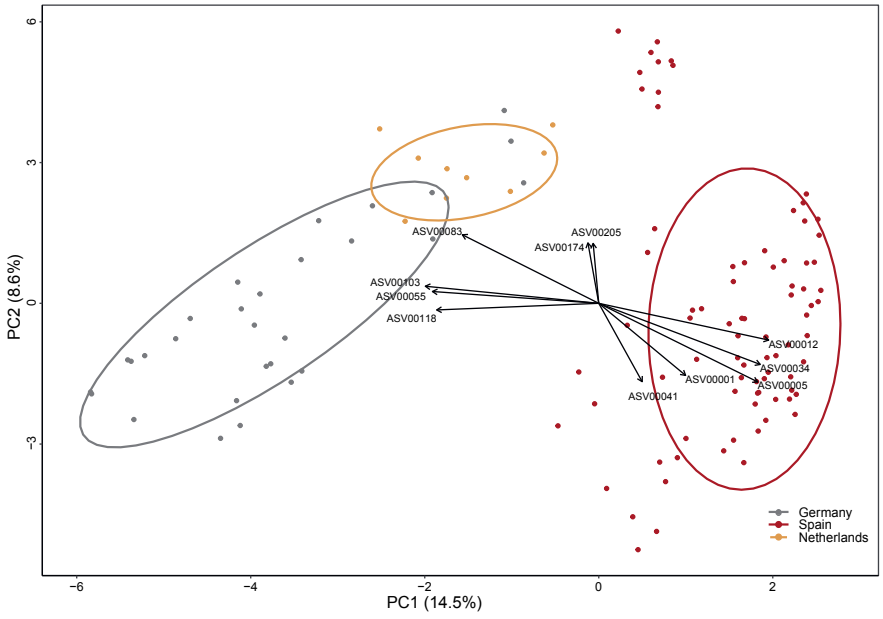


Figure 2. Principal component analysis (PCA) of all samples from the three countries. The two axes explain 14.5% and 8.6% of the total variance, respectively. The dots represent single samples that when plotted close together correspond to observations that have similar scores on the PCA components. The samples clustered per country; red dots represent all samples from Spanish farms on the right side; grey dots on the left side represent samples from German farms; yellow dots in the centre/upper area represent samples from Dutch farms. Each ellipse represents a 75% confidence interval border for each group. The arrows point in the direction of increasing values for the indicated ASVs in the samples.

Culturomics approach

To obtain cultivable bacterial isolates of the piglet tonsil-associated microbiota, swab samples from five piglets from three different farms (ES1, ES2, and NL1) were diluted and plated under different culture conditions and on different media (see Methods section for details). Approximately 10, 000 colonies were screened and random isolates with morphological variation were picked per plate, yielding 518 distinct bacterial isolates representing 60 species of Gram-positive and Gram-negative species from 23 genera (Supplementary Table 1) that were further characterised *in vitro* (Figure 3A). Taxonomic classification was initially based on MALDI-TOF profiles (data not shown) and subsequently validated by full 16S rRNA gene sequencing (Figure 3B/Supplementary Table 1).

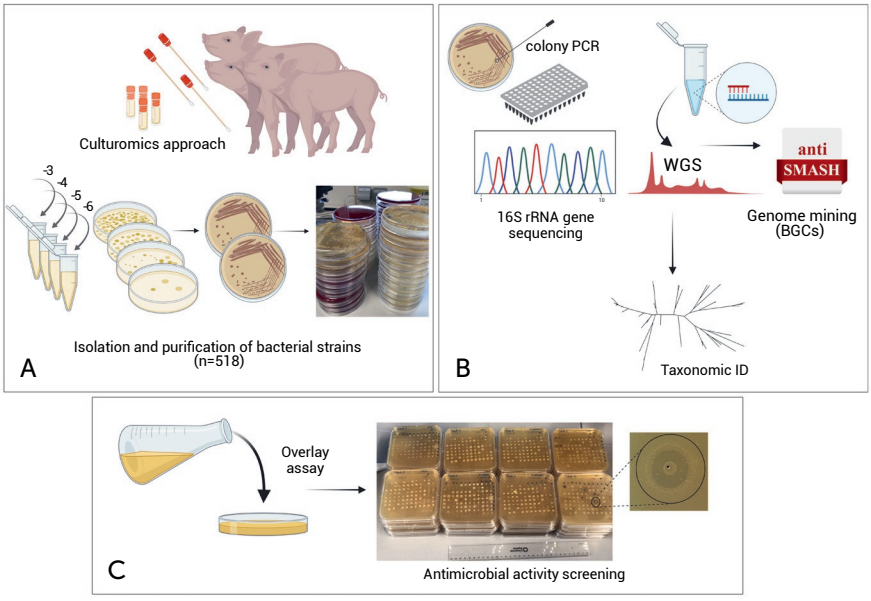


Figure 3. Schematic overview of steps to obtain cultivable species and identify candidate producers of antimicrobial products from the tonsil microbiome. A) Processing of pig tonsil swab samples including isolation and purification steps of bacterial isolates using 9 different culture media. B) Taxonomic identification of pure tonsil isolates using full 16S rRNA gene sequences obtained by colony PCR, and whole genome sequencing (WGS), genome mining and *in silico* prediction of production of bioactive compounds by identification and analysis of biosynthetic gene clusters (BGCs). C) *In vitro* screening for natural antimicrobial compounds produced by tonsil isolates (Supplementary Table 1). Illustration generated using BioRender (https://app.biorender.com).

To gain insight into the relative abundance of these 518 cultured isolates as a fraction of taxonomic diversity present in the total (cultured and uncultured) tonsillar microbiota, the full 16S rRNA gene sequences from the 518 isolates cultured were compared against the 16S rRNA V3-V4 region sequences that we had obtained across the tonsil microbiota samples. The taxonomic diversity of the 518 cultured isolates spanned 23 genera and corresponded to 38.89% of total taxonomic diversity captured by 16S profiling of our tonsillar microbiota dataset. From these cultured isolates, 46.5% (n= 241) were prevalent in >90% of all tonsillar samples that had been obtained at 11 farms from 3 countries. The 16S RNA sequences corresponding to the 255 other cultured isolates had a prevalence between 60% and 20% across tonsillar microbiota samples and were found at relative abundances

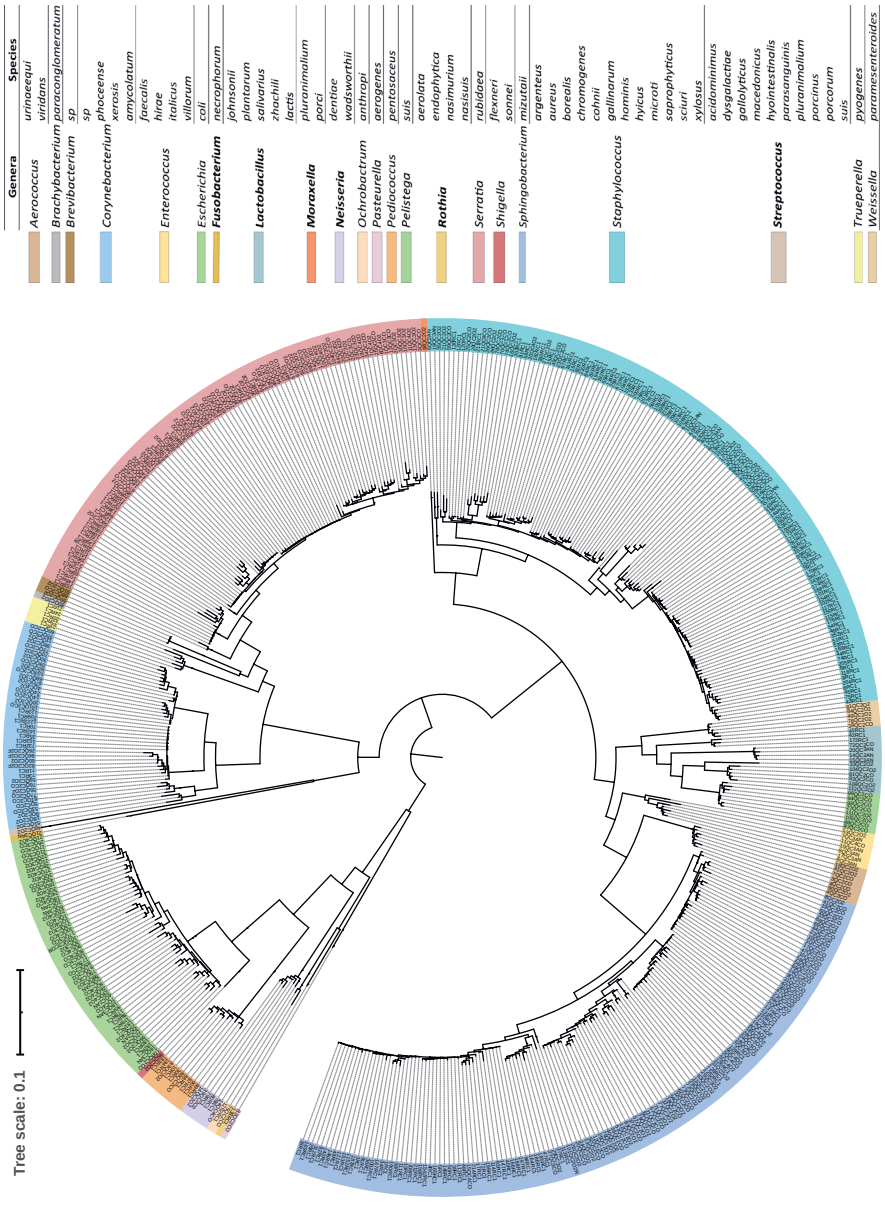


Figure 4. Phylogenetic tree of 518 bacterial single-colony isolates from the porcine tonsillar microbiota based on full 16S rRNA gene sequences. The tree was visualised using the iTOL platform (https://itol.embl.de/). Genera considered part of the core microbiota (90% prevalence and 0.1% abundance threshold) are displayed in bold font.

between 0.4% and 0.05%. The remaining 22 strains were found in the tonsil microbiota at mean relative abundance of ≤0.09% and were thus considered rare. The 16S sequences from cultured isolates which mapped to ASVs of core microbiota members were classified as *Streptococcus suis, Moraxella pluranimalium, Moraxella porci* and *Rothia nasimurium* (Table 2).

Identification of novel species

Based on 16S rRNA gene analysis, genome-based taxonomic assignment, and cellular fatty acids profile we propose two strains as novel species: *Brevibacterium moorei* and *Corynebacterium kozikiae* (Figure 5). *B. moorei* (*Brevibacteriaceae*) (strain 68QC2CO) has a genome length of 3.42 Mbp spanning 69 contigs with an average GC content of 66.76%. Two other strains from our culture collection, 50QC2O2 and 91QC2O2, were also classified as *B. moorei* (Figure 5A). *Brevibacterium rongguiense* 5221 is the closest phylogenetic neighbour, with 97.00% sequence identity to the 16S rRNA gene of *B. moorei*. The Average Nucleotide Identity (ANI) between the three *B. moorei* strains and 21 available genomes of other *Brevibacterium* species varied between 78.45% and 77.15% identity, supporting the notion that *B. moorei* is a separate species (Supplementary Figure 2A). The digital DNA-DNA hybridization (dDDH) sequence identity between *Brevibacterium rongguiense* 5221 and *B. moorei* was 14.8%. The *Brevibacterium* genus was present in low abundance among the 101 sequenced porcine tonsil microbiota samples from different farms and countries (prevalence, 7% among all piglets; mean relative abundance, 8.24E-04%). The major membrane fatty acids isolate of *B. moorei* 68QC2CO are anteiso $C_{17:0}$ (43.92%) and anteiso $C_{15:0}$ (21.74%), iso $C_{15:0}$ (14.60%), and iso $C_{16:0}$ (10.60%), with $C_{16:0}$ (3.67%), $C_{17:0}$ (3.82%) $C_{14:0}$ (0.63%), iso $C_{14:0}$ (0.35%), and $C_{18:0}$ (0.37%) in lower amounts.

The second novel species, *Corynebacterium kozikiae* (isolate 76QC2CO) has a genome length of 2.45 Mbp spanning 57 contigs with an average GC content of 60.36%. Seven other isolates from our culture collection were classified as *C. kozikiae* (strains: 35RC1, 70RC1, 122RC1, 142RC1, 153RC1, 209RC1, 732RC1) (Figure 4B). *C. kozikiae* isolate 76QC2CO has as closest phylogenetic neighbour "unclassified strains *Corynebacterium* sp." (99% rRNA gene identity) followed by *Corynebacterium vitaeruminis* DMS20294 at 97.00% 16S rRNA gene sequence identity. The dDDH identity between *C. vitaeruminis* DMS20294 and *C. kozikiae* was 14.3%. The ANI value between the *C. kozikiae* strains and 80 other reference species from the genus *Corynebacterium* was between 73.79-79.82%, supporting the notion that *C. kozikiae* is a novel species (Supplementary Figure 2B). The mean relative abundance of the genus in the pig tonsil microbiota data from 101 piglets was 0.084%. Major cellular fatty acids of *C. kozikiae* isolate 76QC2CO were $C_{16:0}$ (57.98%), $C_{16:1\ w7c}$

(16.15%), $C_{17:0}$ (5.06%), $C_{18:1\ w9c}$ (6.61%) and $C_{14:0}$ (6.43%). The following fatty acids were detected in lower amounts: $C_{18:0}$ (3.22%) $C_{17:1\ w8c}$ (1.08%), iso $C_{10:0}$ (0.95%), $C_{12:0}$ (0.62%), $C_{14:1\ w5c}$ (0.79%), $C_{9:0}$ (0.56%), 3OH $C_{16:0}$ (0.26%) and $C_{18:2\ w6,9c}$ (0.29%).

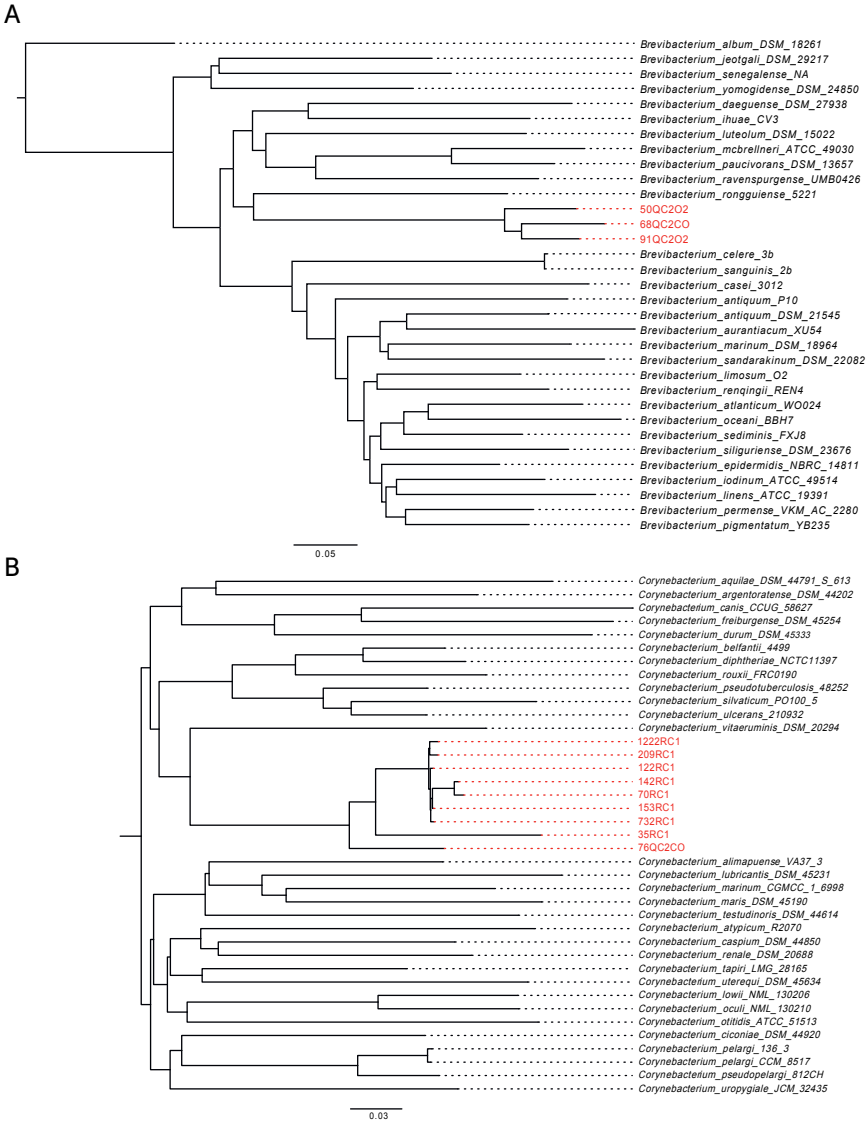


Figure 5. Phylogenetic tree (core genes; see Methods) of the new species **(A)** *B. moorei* and **(B)** *C. kozikiae*. Isolates in red font represent the proposed new species.

Discovery of antagonistic bacteria in overlay inhibition assays and genome mining for antimicrobial peptide-encoding genes

We tested all 518 cultured strains for their growth inhibitory effect on a panel of disease-associated and commensal bacteria using overlay inhibition (antagonism) assays (see Methods). Of these 518 strains, 66 strains reported in this study and 13 strains described in (Chapter IV) (35) showed reproducible inhibitory activity *in vitro* against at least one of the target species tested (Table 3, Figure 3C).

To identify genomic gene clusters predicted to produce natural antibiotics from the tonsillar isolates, whole-genome sequencing was carried out for 45 isolates that had been selected based on 16S rRNA diversity and presence of clear inhibition zones in the antagonism assays (Table 3). These 45 isolates belonged to the genera *Brevibacterium* (n=3), *Corynebacterium* (n=16), *Neisseria* (n=1), *Pediococcus* (n=2), *Pelistega* (n=1), *Staphylococcus* (n=13) and *Streptococcus* (n=9) (Figure 6). To identify biosynthetic gene clusters (BGCs) that might produce natural products, we used the *in silico* analysis tool AntiSMASH (Methods section 6).

In the genomes of the selected 45 isolates 164 BGCs were identified. These were predicted to produce a broad variety of secondary metabolites such as non-ribosomal peptides (NRPs), terpenes, siderophores, polyketides (PKs), bacteriocins, lanthipeptides and ribosomally synthesized and post-translationally modified peptides (RiPPs) (Supplementary Table 2) (Figure 6).

The genomes of seven *Staphylococcus* strains that inhibited growth of *S. suis* (Table 3) contained between 4 and 8 BGCs, some of which were predicted to produce antimicrobial compounds. The BGCs identified in the staphylococcal tonsil isolates included NRPSs and type III polyketide synthases (T3PKSs) and clusters producing RiPPs, siderophores and terpenes. AntiSMASH predicted that specific BGCs identified in *Staphylococcus aureus* produce siderophores with high identity to the siderophores staphyloferrin (87 to 100% identity) and staphylobactin (87% identity) produced by *S. aureus*. The NRPs producing aureusimine that is involved in biofilm production (4) and the bacteriocin hyicin 3682 were identified in some porcine isolates of *S. aureus*. AntiSMASH predicted 5 BGCs in the porcine *S. hyicus* isolates, including clusters with 81% identity to the BGC producing the lanthibiotic suicin 90-1330, and clusters producing the siderophores staphyloferrin (100% identity) and a staphylobactin analogue (51% identity). The genomes of strains from the staphylococcal species *S. borealis, S. cohnii* and *S. saprophyticus*, were predicted to contain four, seven and four BGCs respectively. *S. borealis* strains showed antimicrobial activity against *M. pluranimalium* (Table 3). One of the 4 BGCs predicted in the genome of *S. borealis* had 100% identity to a BGC producing the siderophore staphyloferrin A; the other BGCs appear to be novel.

Table 3. Results of inhibition assays with porcine isolates obtained from tonsil microbiota; shown are strains (ID code) with inhibitory activity against at least one of the target bacteria tested.

ID	16S ID	Inhibition assays									
		SS J28	SS S10	SS P1/7	SP_1 DSM 23759	SP_2 DSM 20725	SP_3 DSM 29126	SA 6538P	EC L4242	PA DSM 10153	MP DSM 22804
3QC2CO	*Aerococcus urinaeequi*	-	-	-	-	-	-	+	-	-	-
4QC2CO	*Aerococcus urinaeequi*	-	-	-	-	-	-	+	-	-	-
7QC2CO	*Aerococcus urinaeequi*	-	-	-	-	-	-	+	-	-	-
15QC2O2	*Aerococcus urinaeequi*	-	-	-	-	-	-	+	-	-	-
4QC3CO	*Aerococcus viridans*	-	-	-	-	-	-	+	-	-	-
90QC2O2	*Aerococcus viridans*	-	-	-	-	-	-	++	+	-	-
76QC2CO	*Corynebacterium sp.(WGS)*	++	-	++	++	-	++	+	-	-	-
20QC2O2	*Corynebacterium sp.(WGS)*	-	-	-	-	-	-	-	-	+/-	+/-
7QC3AN	*Enterococcus faecalis*	-	-	-	+/-	-	+	-	-	-	-
79QC2CO	*Escherichia coli*	-	-	-	-	-	-	-	++	-	-
85QC2CO	*Escherichia coli*	-	-	-	-	-	-	-	++	-	-
97QC2O2	*Escherichia coli*	-	-	-	-	-	-	-	++	-	-
21QC4O2	*Escherichia coli*	-	-	-	-	-	-	-	+	-	-
22QC4O2	*Escherichia coli*	-	-	-	-	-	-	-	+	-	-
1QC2AN	*Escherichia coli*	-	-	-	-	-	-	-	+	-	-
2QC2AN	*Escherichia coli*	-	-	-	-	-	-	-	+	-	-
4QC2AN	*Escherichia coli*	-	-	-	-	-	-	-	+	-	-
16QC3AN	*Escherichia coli*	-	-	-	-	-	-	-	+	-	-
41RC1	*Lactobacillus salivarius*	+/-	-	-	-	-	-	-	-	-	-
172RC1	*Lactobacillus salivarius*	-	-	-	-	+	-	-	-	-	-
1292RC1	*Neisseria dentiae (WGS)*	-	-	-	+/-	-	-	-	-	-	-
105QC2O2	*Pediococcus pentosaceus (WGS)*	-	-	-	-	-	-	-	-	-	+
111QC2O2	*Pediococcus pentosaceus (WGS)*	-	-	-	-	-	-	-	-	-	+
138RC1	*Pelistega suis (WGS)*	-	+/-	-	-	-	-	-	-	-	-
95QC2O2	*Serratia rubidaea*	-	-	-	-	-	-	-	+	-	-
99QC2O2	*Shigella flexneri*	-	-	-	-	-	-	-	+	-	-
16QC3CO	*Shigella sonnei*	-	-	-	-	-	-	-	+	-	-
21QC3CO	*Shigella sonnei*	-	-	-	-	-	-	-	+	-	-

1252RC1	*Staphylococcus aureus*	+/-	-	+	-	-	+	-	-	-	-
128RC1	*Staphylococcus aureus*	+	-	+	+/-	-	-	-	-	-	-
129RC1	*Staphylococcus aureus*	+	-	+	+/-	-	-	-	-	-	-
131RC1	*Staphylococcus aureus (WGS)*	+	-	+	+/-	-	-	-	-	-	-
133RC1	*Staphylococcus aureus (WGS)*	+	-	+	+/-	-	-	-	-	-	-
137RC1	*Staphylococcus aureus (WGS)*	+	-	+	+/-	-	+	-	-	-	-
63QC2O2	*Staphylococcus borealis (WGS)*	-	-	-	-	-	-	-	-	-	+
32QC2O2	*Staphylococcus gallinarum*	-	-	-	-	-	-	-	-	-	+
140RC1	*Staphylococcus hyicus*	-	+/-	-	-	-	-	-	-	-	-
26QC3O2	*Staphylococcus microti*	-	-	-	+/-	-	-	-	-	-	-
41QC2CO	*Staphylococcus sciuri*	+/-	-	-	-	-	-	-	-	-	-
8QC2O2	*Staphylococcus sciuri (WGS)*	-	-	-	-	-	-	-	-	+	+
83QC2O2	*Staphylococcus xylosus*	+/-	-	-	-	-	+	-	-	-	-
12QC3O2	*Streptococcus acidominimus*	+/-	-	-	+/-	-	-	-	-	-	-
8RC1	*Streptococcus gallolyticus*	-	-	-	-	+	-	-	-	-	-
9RC1	*Streptococcus gallolyticus*	-	-	-	-	+	+/-	-	-	-	-
74RC1	*Streptococcus gallolyticus*	-	-	-	-	+	+	-	-	-	-
99RC1	*Streptococcus gallolyticus*	-	-	-	-	+	-	-	-	-	-
150RC1	*Streptococcus gallolyticus*	-	-	-	-	+	-	-	-	-	-
171RC1	*Streptococcus gallolyticus*	-	-	-	-	+	-	-	-	-	-
13QC3AN	*Streptococcus gallolyticus*	+	+	+	-	+	+/-	-	-	-	-
75RC1	*Streptococcus gallolyticus*	-	-	-	-	+	+	-	-	-	-
782RC1	*Streptococcus gallolyticus*	-	-	-	-	+	+	-	-	-	-
143RC1	*Streptococcus gallolyticus*	-	-	-	-	+	-	-	-	-	-
149RC1	*Streptococcus gallolyticus*	-	-	-	-	+	-	-	-	-	-
205RC1	*Streptococcus gallolyticus*	-	-	-	-	+	-	-	-	-	-
151RC1	*Streptococcus gallolyticus (WGS)*	-	-	-	-	+	-	-	-	-	-
12QC3AN	*Streptococcus hyointestinalis*	+	+	+	-	+	-	-	-	-	-
56RC1	*Streptococcus macedonicus*	-	-	-	-	+	-	-	-	-	-
40QC2CO	*Streptococcus parasanguinis*	+/-	-	-	-	-	-	-	-	-	-
5QC2AN	*Streptococcus porcinus*	+	-	+	+/-	-	+/-	-	-	-	-
33QC3O2	*Streptococcus porcorum*	-	-	-	-	-	-	-	+/-	-	-

Inhibition zones were determined after overnight incubation. WGS: Whole genome sequence available. (–) absence of inhibitory activity; (+/-) inhibition zone of 2 mm diameter (weak); (+) inhibition zone between 3 and 5 mm diameter (weak); (++) inhibition zone between 5 and 8 mm diameter. *Streptococcus suis* (SS), *Streptococcus porci* (SP_1) *Streptococcus porcinus* (SP_2) *Streptococcus parasuis* (SP_3), *Escherichia coli* (EC), *Pasteurella aerogenes* (PA), *Moraxella pluranimalium* (MP).

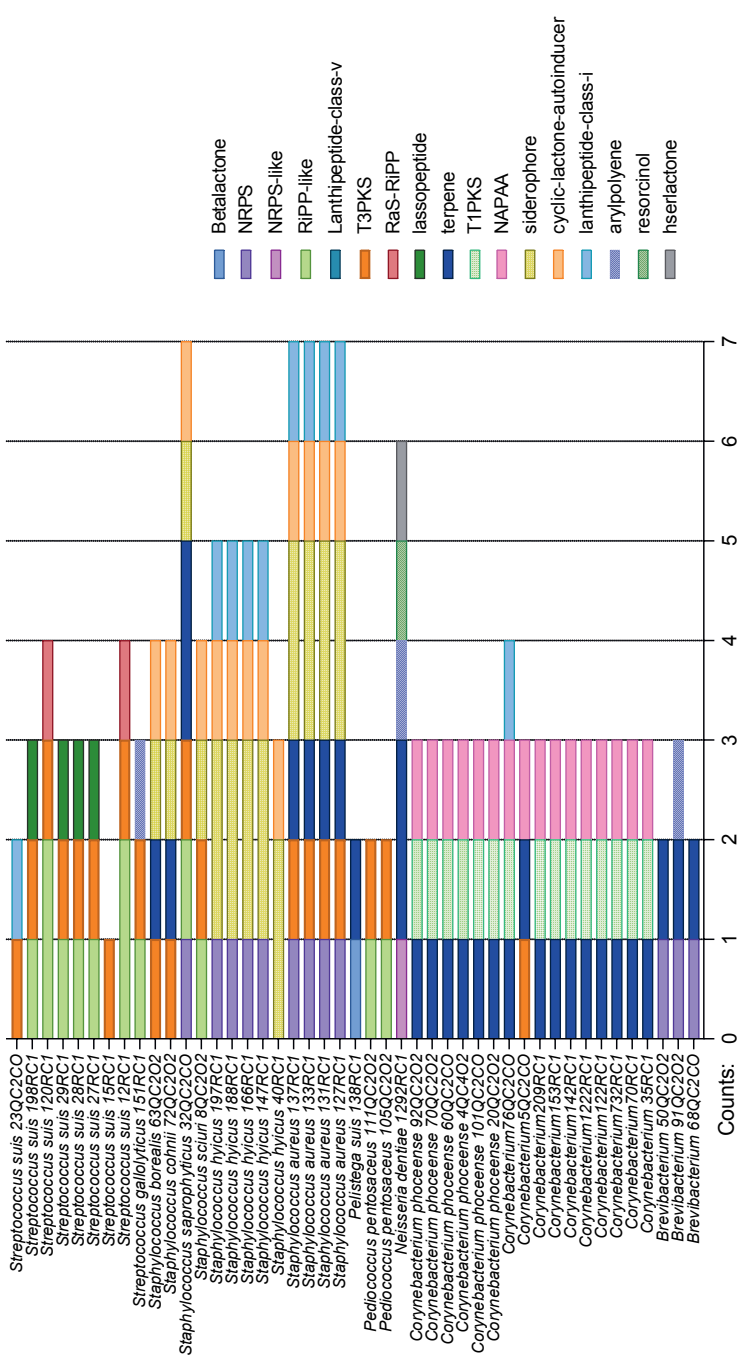


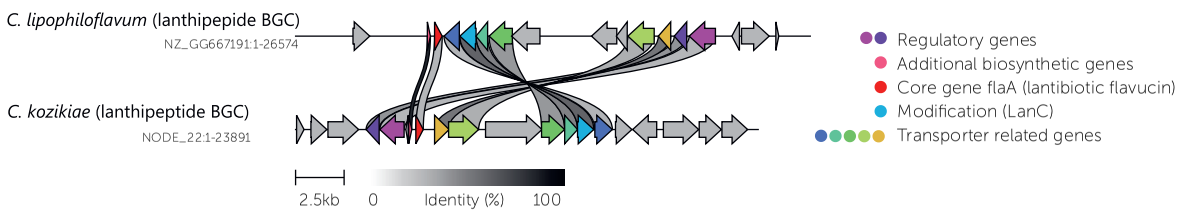
Figure 6. Biosynthetic gene clusters predicted using AntiSMASH from genomes sequences of 45 pig tonsil isolates that had been selected by different colony morphologies and antagonistic activity in bioassays. Different classes of secondary metabolites are represented with different colors.

Out of the sixteen streptococcal species identified in piglet tonsillar microbiota, eight *Streptococcus suis* strains and one *Streptococcus gallolyticus* strain were included for sequencing. The strains from these two species were predicted to produce 10 antimicrobials belonging to different classes including RiPP-like compounds, compounds produced by T3PKSs, lassopeptides and lanthipeptides (Figure 6). T3PKSs were present in all streptococci sequenced in our study. The sequenced *S. suis* genomes carried a gene encoding a radical S-adenosylmethionine (rSAM) enzyme that was predicted by antiSMASH to be involved in biosynthesis of the macrocyclic peptide streptide (75% identity), and the lanthibiotic suicin (36-45% identity). BGCs producing secondary metabolites with 58% identity to Linocin-M18 BGC and with 97.81% similarity to the bacteriocin lactococcin 972 BGC were also identified. Although these molecules have been associated with antibiotic activity (36, 37) none of the sequenced *S. suis* strains showed *in vitro* activity in inhibition assays against the tested targets (Table 3).

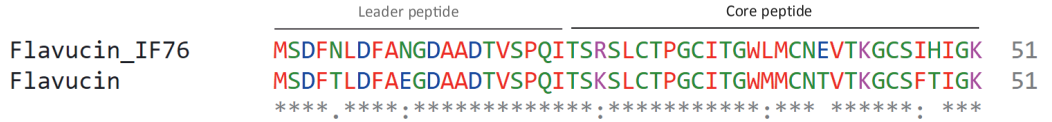
The *Neisseria dentiae* genomes contained BGCs predicted to produce arylpropylene, terpene, homoserine (hser)lactone and RiPP-like compounds. *Pelistega suis* genomes encoded BGCs predicted to produce betalactones and terpenes. *Pediococcus pentosaseus* genomes contained BGCs with high identity to T3PKs and RiPPs (Figure 6). All *Neisseria*, *Pelistega* and *Pediococcus* strains tested had antimicrobial activity in the antagonism assays consistent with the prediction that these BGCs produced natural antibiotic compounds (Table 3). The three strains from the proposed novel species *B. moorei* were predicted to contain highly conserved NRPSs, but no inhibition zones were observed in the inhibition assays using these strains, possibly due to lack of expression *in vitro*.

The genomes of the *Corynebacterium* strains reported in Table 3, including the proposed novel species *C. kozikiae,* were predicted to include NRPSs, T1PKSs, T3PKSs, NAPAA (non-alpha poly-amino acid with NRP domains) and one cluster producing lanthipeptide class I compounds. The isolate 76QC2CO (*C. kozikiae*), was predicted to produce a class I lanthipeptide and displayed strong and consistent inhibitory activity *in vitro* (Table 3). In agar overlay- and agar well diffusion assays, isolate 76QC2CO inhibited growth of multiple strains and serotypes of the porcine pathogen *S. suis* and two other closely related streptococci, as well as *S. aureus*. AntiSMASH detected a lanthipeptide class I BGC predicted to synthesize a compound 94% similar to the lantibiotic flavucin, previously identified in the genome of *Corynebacterium lipophiloflavum* DSM 44291 (38) (Fig 7A). Compared to the original flavucin produced by *C. lipophiloflavum* DSM 44291, the predicted lantibiotic of isolate 76QC2CO contains two amino acid modifications in the leader peptide and five in the core peptide (Figure 7B); these post-translational modifications

A


C. lipophiloflavum (lanthipepide BGC)
NZ_GG667191:1-26574
C. kozikiae (lanthipeptide BGC)
NODE_22:1-23891
2.5kb
0
Identity (%)
100
Regulatory genes
Additional biosynthetic genes
Core gene flaA (lantibiotic flavucin)
Modification (LanC)
Transporter related genes

B


Leader peptide
Core peptide
Flavucin_IF76 MSDFNLDFANGDAADTVSPQITSRSLCTPGCITGWLMCNEVTKGCSIHIGK 51
Flavucin MSDFTLDFAEGDAADTVSPQITSKSLCTPGCITGWMMCNTVTKGCSFTIGK 51

C

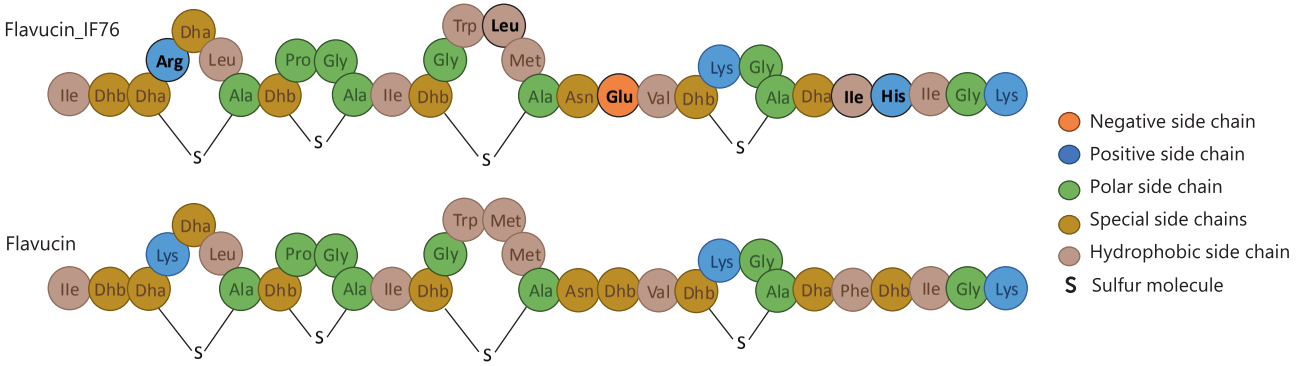

Flavucin_IF76
Flavucin
Negative side chain
Positive side chain
Polar side chain
Special side chains
Hydrophobic side chain
S Sulfur molecule

Figure 7. Summary of the major features of flavucin IF76, a novel variant of the class I lanthipeptide Flavucin. **(A)** Conserved genes present in the biosynthetic gene clusters (BGCs) producing the class I lanthipeptide of the species *Corynebacterium kozikiae* and *C. lipophiloflavum* predicted to produce flavucin; homologous genes from the flavucin IF76 and original flavucin BGCs are labeled with the same color. Cluster organization was visualized using Clinker (https://github.com/gamcil/clinker). **(B)** Amino acid sequences of the two variant flavucin peptides produced by the genomes of *C. kozikiae* and *C. lipophiloflavum*, displaying the two modifications present in the leader peptide and the five modifications present in the core peptide. **(C)** Schematic representation of the structure of lanthipeptides flavucin IF76 and the original flavucin; the chains of amino acid residues containing the five amino acid differences are highlighted in black.

may lead to a mature peptide carrying five amino acid differences compared to the original flavucin. We named this novel flavucin variant flavucin IF76. The predicted modifications were mainly due to substitutions of amino acids with similar biochemical properties, apart from a mutation yielding a different amino acid with a side chain of different size. The impact of this alterations displayed in the structure of flavucin IF76 may result in differences in 3D folding and/or in the antimicrobial activity compared to the known flavucin (Figure 7C).

We obtained and sequenced 9 isolates of the proposed novel species *C. kozikiae* (Figure 5) and found that only *C. kozikiae* isolate 76QC2CO contained the lanthipeptide class I BGC predicted to produce flavucin IF76. Flavucin IF76 is stable at high temperatures (>95 °C), and proteinase K and trypsin treatments had small effect (<10%) on peptide antimicrobial activity. Moreover, flavucin IF76 displayed no cytotoxicity to HEK-293 and HEPG2 mammalian cell lines (unpublished data).

Finally, strains from the streptococcal species *S. macedonicus, S. porcinus, S. porcorum* and strains belonging to the genera *Aerococcus, Enterococcus, Lactobacillus* and *Escherichia/Shigella* also showed inhibitory activity against the target panel of pathogens (Table 3); we did not further investigate these isolates because their spectrum of antagonism did not go appreciably far beyond the activity of other isolates that we had selected to characterize in greater detail.

Discussion

To assess taxonomic diversity in tonsil microbiota from healthy piglets at 11 farms in Germany, Spain and The Netherlands, we used two complementary methods to characterise the tonsillar bacterial diversity. Using 16S rRNA gene amplicon sequencing, we discovered a core tonsil-associated microbiota that consisted of 14 genera and 17 individual amplicon sequence variants (ASVs) at more than 0.1% abundance in 90% or more of the sampled healthy piglets. By culturomics, we obtained 218 strains belonging to 6 of the 14 core genera. The bacterial taxa that we cultured and identified by 16S rRNA gene profiling were largely consistent with earlier DNA-based taxonogenomic studies on healthy piglets without antibiotic treatment (39, 40), underpinning the relevance of these taxa in the establishment of a health-associated early life microbiome of piglets. The core genera that we found in the 101 piglets have been reported by others to be members of the tonsil microbiota of healthy piglets with no recent history of respiratory disease (41), reinforcing the idea they are associated with a healthy microbiota and might be symbionts. Other genera we uncovered at lower prevalence (≤16%) and abundance (≤0.1%) included *Pasteurella*, *Haemophilus* and *Peptostreptococcus*, genera also previously reported to be core members of the tonsil microbiota of piglets. However, some species within these genera contain disease-associated strains causing infection in different ages and with host in compromised health status (42). Compared to other studies, we observed minor differences in the abundance of certain genera (Figure 1), and these differences might relate to the transport of pigs, country- and/or farm-specific differences in antimicrobial usage, animal feed compositions, and various environmental conditions and farming practices.

We isolated 518 bacterial pure cultures representing 60 species from 23 genera that were identified by their corresponding 16S rRNA gene sequences from five palatine tonsil samples randomly taken from healthy piglets on 11 farms from ES, DE and NL. Our collection of isolates covered a substantial proportion of the high bacterial biodiversity associated with porcine tonsil surface even though we did not use selective medium and only a few different culture conditions (10). We discovered two novel low abundance species belonging to the genera *Corynebacterium* and *Brevibacterium* that harboured BGCs predicted to produce bio-active metabolites likely involved in microbiota ecology.

Our cultured bacterial tonsil collection contained aerobic and facultative anaerobic bacteria, of which 86% are Gram-positive bacteria from the phyla Firmicutes (new name: Bacillota) and Actinobacteria (new name: Actinomycetota), and 14% are Gram-negative species from the phyla Proteobacteria (new name: Pseudo-

monadota) and Bacteroidetes (new name: Bacteroidota). Our collection did not include members of the genus *Actinobacillus,* even though it is one the most abundant genera in the tonsil microbiota. The failure to culture species of *Actinobacillus* is probably due to their fastidious growth requirements and the long incubation times needed for colony formation (43) which were not included in our methods.

Strains belonging to the genera *Moraxella, Streptococcus, Fusobacterium, Lactobacillus, Rothia* and *Neisseria* were present in the microbiota in ≥90% of all 101 tonsil samples, and species from these genera cultured comprised 71% of our collection. These microbiota members might be associated with relevant ecological interactions within the niche. Therefore our collection of sequenced and biologically characterized strains is a valuable resource for future research on the health-associated tonsillar microbiome. The remainder of the cultured strains (29%) featured low-abundance ASVs (<0.1% relative abundance). Although we focused on characterizing the most abundant taxa for practical and applied reasons, the transient or less abundant species may also contribute to establishment of health-associated microbial communities through several mechanisms such as, blocking invasion of niches by disease-associated species, (thus avoiding dysbiosis), and contributing to microbiota metabolic potential by increasing genetic and enzymatic diversity within the population (44, 45).

The microbiota of human and animal body sites has been previously recognized as a source of potentially novel and bioactive secondary metabolites (7, 10). The ability to produce natural products can provide adaptive and competitive advantages within different environments, and strains producing interesting bio-active metabolites may form a relevant resource for pharmaceutical drug discovery (46). Whole genome sequencing of 45 strains belonging to 13 species from the genera *Brevibacterium, Corynebacterium, Streptococcus, Staphylococcus, Escherichia, Neisseria, Pelistega* and *Pediocuccus* revealed the presence of BGCs, predicted to produce compounds with antimicrobial activities. Twelve of these 45 strains displayed inhibitory activity *in vitro* (Table 3; Figure 6). Eight of the species carrying BGCs predicted to produce antibacterials did not show any inhibitory activity. This could be due to lack of expression under our culture conditions, lack of activity against the target bacteria tested in our study or simply because the produced secondary metabolites had other functions than antimicrobial activity (47). The proposed novel species *Corynebacterium kozikiae* strain 76QC2CO is a promising example of the potential of the tonsil microbiota in the discovery of commensal isolates able to antagonize pathogens as *S. suis*. *C. kozikiae* strain 76QC2CO was predicted to produce the lanthipeptide flavucin variant IF76 and showed consistent

in vitro antimicrobial activity against five of the Gram-positive target bacteria (Table 3) and other features as thermo stability and no cytotoxicity to mammalian cells.

Sixty-two BGCs including NRPSs, T3PKSs and clusters producing siderophores, were identified in all five species of *Staphylococcus* that we cultured. *S. aureus* and *S. hyicus* include opportunistic pathogenic strains that reside in the upper respiratory tract and skin of pigs (48, 49). Both species have been associated with production of antimicrobials with activity against other (pathogenic) bacteria, and we observed that our cultured isolates from these species showed inhibitory activity in our antagonism screens (Table 3). In the genome sequences from our cultured *S. aureus* strains, AntiSMASH predicted clusters producing the siderophores staphyloferrin and staphylobactin. Production of siderophores in *S. aureus* increased survival rates of *S. aureus* strains in iron-deficient environments compared to mutants lacking the genes necessary for siderophore production (50) and provided adaptive advantages in the colonization of niches low in iron (50). Some *S. aureus* strains also produce class II bacteriocins such as aureocin and lanthibiotics (Bac1829, C55a/b and MP1102) which are active against a wide range of Gram-positive and Gram-negative bacteria including other staphylococci, *S. suis, C. pseudotuberculosis, H. parasuis* and *P. multocida* (51-53). Twenty-six other BGCs were predicted in eight *S. suis* strains and one *S. gallolyticus* strain from our culture collection (Figure 7). S-adenosyl-L-methionine (rSAM), a RiPP-associated radical molecule that had been identified by AntiSMASH in most genomes from these 9 streptocccal strains, may be involved in the biosynthesis of natural products that function as chemical messengers in *quorum sensing* and intraspecies communication (54, 55).

Most of the genomes from our cultured tonsillar microbiota isolates with *in vitro* antimicrobial activity contained BGCs that had previously been described in microbiota from animal and human body sites (Supplementary Table 2). Nonetheless, 68.6% of BGCs identified in the genomes from tonsillar bacteria that we cultured had less than 10% homology, or no homology with known BGCs. The natural products produced by these BGCs might provide lead molecules that could be developed into novel therapeutic molecules.

Conclusions

The tonsil of piglets carries an extensive and diverse microbiota. Antagonism assays of cultured tonsillar isolates and mining of genomes of selected strains displaying reproducible antagonism showed that porcine tonsil microbiota include isolates that produce secondary metabolites involved in competition interference of with

opportunistic pathogens. The genomes from these antagonistic taxa contained novel BGCs which were predicted to produce various (so far unidentified) natural bioactive products that might have antimicrobial activity. These results give insights in the unexplored biosynthetic capacity of the tonsil microbiota. The chemical and functional characterization of the natural compounds produced by these cultivable antagonistic strains could provide leads in the development of novel antimicrobials and may eventually lead to new antibacterial molecules for treatment of infectious diseases.

List of abbreviations

ASV: Amplicon sequence variants; ANI: Average Nucleotide Identity; BGCs: biosynthetic gene clusters; dDDH: DNA-DNA hybridization; DE: Germany; NL: The Netherlands; ES: Spain; WGS: whole genome sequencing, PCA: Principal Coordinate Analysis, NRPSs: non-ribosomal synthetized peptides, RiPPs: ribosomally synthesized and post-translationally modified peptides, TPKSs: polyketide synthases, MALDI-TOF: Matrix assisted laser desorption/ionisation - time of flight mass spectrometry, PRDC:porcine respiratory disease complex, NAPAA: non-alpha poly-amino acid with NRP domains; PERMANOVA: Permutational analysis of variance; rSAM: radical S-adenosylmethionine.

Ethical statement

This study uses samples obtained for diagnostic procedures performed according to the ethical principles and guidelines covered by EU Directive 2010/63/EU.

Availability of data and material

The sequencing data are available at NCBI under the following BioProjects: PRJNA862074 forthewhole-genomesequencing(acessions:JANIJX000000000-JANILP000000000), PRJNA854341 for the microbiota sequencing data and the 16S rRNA gene sequencing of the cultured isolates are available under the accession GenBank numbers: OP067681-OP068110. All data generated for this study are included in the manuscript and/or in the Supplementary Files.

Authors' contributions

I.M.F.O., P.v.B., and J.M.W. conceptualized the study. I.M.F.O. and S.F., performed the data analysis and interpretation of results under the supervision of M.F.G., H.H, J.B., P.v.B. and J.M.W. The experiments and culturomic approach were performed by I.M.F.O. The microbiota analyses performed by S.F. The manuscript was written by I.M.F.O., P.v.B., J.M.W with input from all co-authors. All authors contributed to the article and approved the final manuscript.

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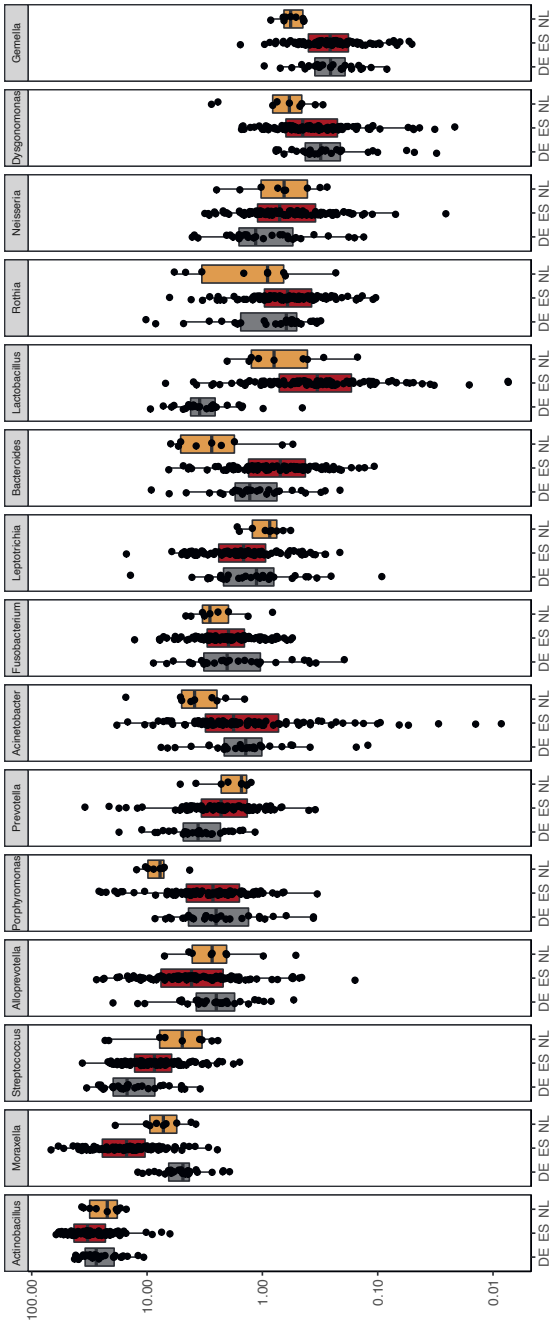
Supplementary Material



Supplementary Figure 1. The Shannon alpha-diversity of the core genera microbiota within countries. Spain (ES); Germany (DE); Netherlands (NL).

Supplementary Table 1. Collection of bacterial isolates of the piglet tonsil-associated microbiota (n=518).

							Inhibition assays								
Farm	Pig age	Medium	Atm	ID	WGS	16S ID	Gram	*Streptococcus suis J28*	*Streptococcus suis S10*	*Streptococcus suis P1/7*	*Streptococcus porci DSM 23759*	*Streptococcus porcinus DSM 20725*	*Streptococcus parasuis DSM 29126*	*Staphylococcus aureus L100*	*Escherichia coli L4242*	*Pasteurella aerogenes DMS 21448*	*Moraxella pluranimalium DSM 22804*
ES1	-1 week	BHI	O_2	1RC1		*Staphylococcus hyicus*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	BHI	O_2	2RC1		*Streptococcus gallolyticus*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	BHI	O_2	3RC1		*Staphylococcus hyicus*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	BHI	O_2	4RC1		*Staphylococcus hyicus*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	BHI	O_2	5RC1		*Staphylococcus hyicus*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	BHI	O_2	6RC1		*Streptococcus gallolyticus*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	BHI	O_2	7RC1		*Streptococcus gallolyticus*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	BHI	O_2	8RC1		*Streptococcus gallolyticus*	+	-	-	-	-	+	-	-	-	-	-
ES1	-1 week	BHI	O_2	9RC1		*Streptococcus gallolyticus*	+	-	-	-	-	+	+/-	-	-	-	-
ES1	-1 week	BHI	O_2	10RC1		*Pasteurella aerogenes*	-	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	BHI	O_2	11RC1		*Rothia nasisuis*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	BHI	O_2	12RC1	yes	*Streptococcus suis*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	BHI	O_2	13RC1		*Streptococcus pluranimalium*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	BHI	O_2	15RC1	yes	*Streptococcus suis*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	BHI	O_2	16RC1		*Streptococcus suis*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	SBA	O_2	17RC1		*Staphylococcus hyicus*	+	-	-	-	-	-	-	-	-	-	-

Supplementary Table 1. Continued.

								Inhibition assays										
Farm	Pig age	Medium	Atm	ID	WGS	16S ID	Gram	*Streptococcus suis J28*	*Streptococcus suis S10*	*Streptococcus suis P1/7*	*Streptococcus porci DSM 23759*	*Streptococcus porcinus DSM 20725*	*Streptococcus parasuis DSM 29126*	*Staphylococcus aureus L100*	*Escherichia coli L4242*	*Pasteurella aerogenes DMS 21448*	*Moraxella pluranimalium DSM 22804*
ES1	-1 week	SBA	O_2	18RC1		*Staphylococcus hyicus*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	SBA	O_2	19RC1		*Staphylococcus hyicus*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	SBA	O_2	20RC1		*Trueperella pyogenes*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	SBA	O_2	21RC1		*Trueperella pyogenes*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	SBA	O_2	22RC1		*Trueperella pyogenes*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	SBA	O_2	23RC1		*Trueperella pyogenes*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	SBA	O_2	24RC1		*Trueperella pyogenes*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	SBA	O_2	25RC1		*Streptococcus suis*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	SBA	O_2	26RC1		*Staphylococcus hyicus*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	SBA	O_2	27RC1	yes	*Streptococcus suis*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	SBA	O_2	28RC1	yes	*Streptococcus suis*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	SBA	O_2	29RC1	yes	*Streptococcus suis*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	SBA	O_2	30RC1		*Pasteurella aerogenes*	-	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	SBA	O_2	31RC1	yes	*Rothia nasisuis*	+	+	+/-	+	+	+	+	-	-	-	-
ES1	-1 week	SBA	O_2	312RC1		*Staphylococcus hyicus*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	LB	O_2	33RC1		*Rothia nasisuis*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	LB	O_2	34RC1		*Staphylococcus hyicus*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	LB	O_2	35RC1	yes	*Corynebacterium kozikiae*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	LB	O_2	352RC1		*Streptococcus gallolyticus/macedonicus*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	LB	O_2	36RC1		*Staphylococcus hyicus*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	LB	O_2	37RC1		*Staphylococcus chromogenes*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	LB	O_2	38RC1		*Staphylococcus hyicus*	+	-	-	-	-	-	-	-	-	-	-

ES1	-1 week	LB	O_2	39RC1		*Staphylococcus hyicus*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	LB	O_2	40RC1	yes	*Staphylococcus hyicus*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	LB	O_2	41RC1		*Lactobacillus salivarius*	+	+/-	-	-	-	-	-	-	-	-	-
ES1	-1 week	LB	O_2	42RC1		*Lactobacillus salivarius*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	LB	O_2	43RC1		*Streptococcus gallolyticus*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	LB	O_2	44RC1		*Streptococcus gallolyticus*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	LB	O_2	45RC1		*Streptococcus gallolyticus*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	LB	O_2	46RC1		*Streptococcus gallolyticus*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	MH	O_2	47RC1		*Staphylococcus chromogenes*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	MH	O_2	49RC1		*Staphylococcus hyicus*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	MH	O_2	48RC1		*Staphylococcus hyicus*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	MH	O_2	50RC1		*Staphylococcus hyicus*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	MH	O_2	51RC1		*Streptococcus gallolyticus*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	MH	O_2	52RC1		*Streptococcus gallolyticus*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	MH	O_2	53RC1		*Streptococcus gallolyticus*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	MH	O_2	54RC1		*Streptococcus gallolyticus*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	MH	O_2	55RC1		*Streptococcus suis*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	MH	O_2	56RC1		*Streptococcus macedonicus*	+	-	-	-	-	+	-	-	-	-	-
ES1	-1 week	MH	O_2	57RC1		*Streptococcus suis*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	MH	O_2	58RC1		*Streptococcus suis*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	MH	O_2	59RC1		*Streptococcus suis*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	MH	O_2	60RC1		*Streptococcus suis*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	MH	O_2	62RC1		*Streptococcus suis*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	MH	O_2	63RC1	yes	*Rothia nasimurium*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	MH	O_2	64RC1		*Rothia nasimurium*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	THB	O_2	65RC1	yes	*Rothia nasimurium*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	THB	O_2	652RC1	yes	*Rothia nasimurium*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	THB	O_2	66RC1	yes	*Rothia nasimurium*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	THB	O_2	67RC1	yes	*Rothia nasimurium*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	THB	O_2	672RC1		*Streptococcus suis*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	THB	O_2	673RC1	yes	*Rothia nasimurium*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	THB	O_2	68RC1	yes	*Rothia nasimurium*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	THB	O_2	682RC1		*Streptococcus suis*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	THB	O_2	69RC1	yes	*Rothia nasisuis*	+	-	-	+	+	+	+	-	-	-	-
ES1	-1 week	THB	O_2	70RC1	yes	*Corynebacterium kozikiae*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	THB	O_2	71RC1		*Corynebacterium kozikiae*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	THB	O_2	72RC1		*Staphylococcus hyicus*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	THB	O_2	722RC1		*Rothia nasisuis*	+	-	-	-	-	-	-	-	-	-	-

Supplementary Table 1. Continued.

								Inhibition assays									
Farm	Pig age	Medium	Atm	ID	WGS	16S ID	Gram	*Streptococcus suis J28*	*Streptococcus suis S10*	*Streptococcus suis P1/7*	*Streptococcus porci DSM 23759*	*Streptococcus porcinus DSM 20725*	*Streptococcus parasuis DSM 29126*	*Staphylococcus aureus L100*	*Escherichia coli L4242*	*Pasteurella aerogenes DMS 21448*	*Moraxella pluranimalium DSM 22804*
ES1	-1 week	THB	O_2	73RC1		*Corynebacterium kozikiae*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	THB	O_2	732RC1	yes	*Corynebacterium kozikiae*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	THB	O_2	74RC1		*Streptococcus gallolyticus*	+	-	-	-	-	+	+	-	-	-	-
ES1	-1 week	THB	O_2	75RC1		*Streptococcus gallolyticus*	+	-	-	-	-	+	+	-	-	-	-
ES1	-1 week	THB	O_2	76RC1		*Streptococcus suis*	+	-	-	-	-	+	+	-	-	-	-
ES1	-1 week	THB	O_2	77RC1		*Streptococcus suis*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	THB	O_2	78RC1		*Moraxella pluranimalium*	-	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	THB	O_2	782RC1		*Streptococcus gallolyticus*	+	-	-	-	-	+	+	-	-	-	-
ES1	-1 week	THB	O_2	79RC1		*Moraxella porci*	-	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	THB	O_2	80RC1		*Moraxella pluranimalium*	-	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	THB	O_2	81RC1		*Streptococcus suis*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	THB	O_2	82RC1		*Streptococcus suis*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	THB	O_2	84RC1		*Streptococcus suis*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	MC	O_2	85RC1		*Escherichia coli*	-	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	MC	O_2	86RC1		*Escherichia coli*	-	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	MC	O_2	87RC1		*Pasteurella aerogenes*	-	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	MC	O_2	88RC1		*Pasteurella aerogenes*	-	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	MC	O_2	89RC1		*Pasteurella aerogenes*	-	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	MC	O_2	90RC1		*Pasteurella aerogenes*	-	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	M17	O_2	91RC1		*Staphylococcus hyicus*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	M17	O_2	92RC1		*Staphylococcus hyicus*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	M17	O_2	93RC1		*Staphylococcus hyicus*	+	-	-	-	-	-	-	-	-	-	-

ES1	-1 week	M17	O_2	94RC1		*Staphylococcus chromogenes*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	M17	O_2	97RC1		*Streptococcus suis*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	M17	O_2	99RC1		*Streptococcus gallolyticus*	+	-	-	-	-	+	-	-	-	-	-
ES1	-1 week	M17	O_2	100RC1		*Streptococcus gallolyticus*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	M17	O_2	1002RC1		*Staphylococcus hyicus*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	M17	O_2	101RC1		*Streptococcus gallolyticus*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	M17	O_2	102RC1		*Streptococcus gallolyticus*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	M17	O_2	103RC1		*Streptococcus suis*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	M17	O_2	104RC1		*Streptococcus suis*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	M17	O_2	105RC1		*Streptococcus suis*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	M17	O_2	106RC1		*Streptococcus suis*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	M17	O_2	107RC1	yes	*Rothia nasisuis*	+	+	-	-	-	-	-	-	-	-	-
ES1	-1 week	M17	O_2	108RC1		*Rothia nasisuis*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	M17	O_2	109RC1		*Rothia nasisuis*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	M17	O_2	110RC1	yes	*Rothia nasimurium*	+	+/-	-	-	+/-	-	-	-	-	-	-
ES1	-1 week	BHI	CO_2	111RC1		*Staphylococcus hyicus*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	BHI	CO_2	112RC1		*Staphylococcus hyicus*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	BHI	CO_2	113RC1		*Staphylococcus chromogenes*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	BHI	CO_2	114RC1		*Staphylococcus hyicus*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	BHI	CO_2	115RC1		*Streptococcus gallolyticus*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	BHI	CO_2	1152RC1		*Staphylococcus hyicus*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	BHI	CO_2	116RC1		*Streptococcus gallolyticus*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	BHI	CO_2	117RC1		*Streptococcus gallolyticus*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	BHI	CO_2	118RC1		*Streptococcus gallolyticus*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	BHI	CO_2	120RC1	yes	*Streptococcus suis*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	BHI	CO_2	121RC1		*Staphylococcus hyicus*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	BHI	CO_2	122RC1	yes	*Corynebacterium kozikiae*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	BHI	CO_2	1222RC1	yes	*Corynebacterium kozikiae*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	BHI	CO_2	123RC1	yes	*Rothia nasimurium*	+	+/-	-	-	+/-	-	-	-	-	-	-
ES1	-1 week	BHI	CO_2	124RC1	yes	*Rothia nasimurium*	+	+/-	-	-	+/-	-	-	-	-	-	-
ES1	-1 week	BHI	CO_2	125RC1		*Rothia nasimurium*	+	+/-	-	-	+/-	-	-	-	-	-	-
ES1	-1 week	BHI	CO_2	1252RC1		*Staphylococcus aureus*	+	+/-	-	+	-	-	+	-	-	-	-
ES1	-1 week	SBA	CO_2	126RC1		*Staphylococcus aureus*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	SBA	CO_2	127RC1	yes	*Staphylococcus aureus*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	SBA	CO_2	128RC1		*Staphylococcus aureus*	+	+	-	+	+/-	-	-	-	-	-	-
ES1	-1 week	SBA	CO_2	129RC1		*Staphylococcus aureus*	+	+	-	+	+/-	-	-	-	-	-	-
ES1	-1 week	SBA	CO_2	1292RC1	yes	*Neisseria dentiae*	-	-	-	-	+/-	-	-	-	-	-	-
ES1	-1 week	SBA	CO_2	130RC1		*Streptococcus suis*	+	-	-	-	-	-	-	-	-	-	-

Supplementary Table 1. Continued.

								Inhibition assays									
Farm	Pig age	Medium	Atm	ID	WGS	16S ID	Gram	*Streptococcus suis J28*	*Streptococcus suis S10*	*Streptococcus suis P1/7*	*Streptococcus porci DSM 23759*	*Streptococcus porcinus DSM 20725*	*Streptococcus parasuis DSM 29126*	*Staphylococcus aureus L100*	*Escherichia coli L4242*	*Pasteurella aerogenes DMS 21448*	*Moraxella pluranimalium DSM 22804*
ES1	-1 week	SBA	CO_2	131RC1	yes	*Staphylococcus aureus*	+	+	-	+	+/-	-	-	-	-	-	-
ES1	-1 week	SBA	CO_2	132RC1		*Streptococcus suis*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	SBA	CO_2	133RC1	yes	*Staphylococcus aureus*	+	+	-	+	+/-	-	-	-	-	-	-
ES1	-1 week	SBA	CO_2	134RC1		*Streptococcus suis*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	SBA	CO_2	135RC1		*Streptococcus suis*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	SBA	CO_2	136RC1	yes	*Rothia nasimurium*	+	-	-	-	+/-	-	-	-	-	-	-
ES1	-1 week	SBA	CO_2	137RC1	yes	*Staphylococcus aureus*	+	+	-	+	+/-	-	+	-	-	-	-
ES1	-1 week	SBA	CO_2	139RC1		*Rothia nasisuis*	+	-	+/-	-	-	-	-	-	-	-	-
ES1	-1 week	SBA	CO_2	138RC1	yes	*Pelistega suis*	-	-	+/-	-	-	-	-	-	-	-	-
ES1	-1 week	SBA	CO_2	140RC1		*Staphylococcus hyicus*	+	-	+/-	-	-	-	-	-	-	-	-
ES1	-1 week	SBA	CO_2	141RC1	yes	*Rothia nasisuis*	+	+	+/-	+	+	+	+	-	-	-	-
ES1	-1 week	SBA	CO_2	142RC1	yes	*Corynebacterium kozikiae*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	SBA	CO_2	143RC1		*Streptococcus gallolyticus*	+	-	-	-	-	+	-	-	-	-	-
ES1	-1 week	SBA	CO_2	144RC1		*Streptococcus gallolyticus*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	LB	CO_2	145RC1		*Staphylococcus hyicus*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	LB	CO_2	146RC1		*Staphylococcus hyicus*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	LB	CO_2	147RC1	yes	*Staphylococcus hyicus*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	LB	CO_2	148RC1		*Rothia aerolata*	+	+	-	-	-	-	-	-	-	-	-
ES1	-1 week	LB	CO_2	149RC1		*Streptococcus gallolyticus*	+	-	-	-	-	+	-	-	-	-	-
ES1	-1 week	LB	CO_2	150RC1		*Streptococcus gallolyticus*	+	-	-	-	-	+	-	-	-	-	-
ES1	-1 week	LB	CO_2	151RC1	yes	*Streptococcus gallolyticus*	+	-	-	-	-	+	-	-	-	-	-
ES1	-1 week	LB	CO_2	152RC1	yes	*Rothia nasisuis*	+	+	+/-	+	+	+	+	-	-	-	-

ES1	-1 week	LB	CO_2	1522RC1		*Streptococcus gallolyticus*	+	-	-	-	-	-	-	-	-
ES1	-1 week	LB	CO_2	153RC1	yes	*Corynebacterium kozikiae*	+	-	-	-	-	-	-	-	-
ES1	-1 week	LB	CO_2	154RC1		*Streptococcus gallolyticus*	+	-	-	-	-	-	-	-	-
ES1	-1 week	LB	CO_2	1542RC1		*Staphylococcus hyicus*	+	-	-	-	-	-	-	-	-
ES1	-1 week	LB	CO_2	155RC1		*Streptococcus gallolyticus*	+	-	-	-	-	-	-	-	-
ES1	-1 week	LB	CO_2	156RC1		*Streptococcus gallolyticus*	+	-	-	-	-	-	-	-	-
ES1	-1 week	LB	CO_2	157RC1		*Streptococcus gallolyticus*	+	-	-	-	-	-	-	-	-
ES1	-1 week	LB	CO_2	158RC1		*Staphylococcus hyicus*	+	-	-	-	-	-	-	-	-
ES1	-1 week	MH	CO_2	159RC1	yes	*Rothia aerolata*	+	-	-	-	-	-	-	-	-
ES1	-1 week	MH	CO_2	160RC1		*Streptococcus suis*	+	-	-	-	-	-	-	-	-
ES1	-1 week	MH	CO_2	161RC1		*Streptococcus suis*	+	-	-	-	-	-	-	-	-
ES1	-1 week	MH	CO_2	162RC1		*Streptococcus suis*	+	-	-	-	-	-	-	-	-
ES1	-1 week	MH	CO_2	163RC1		*Staphylococcus hyicus*	+	-	-	-	-	-	-	-	-
ES1	-1 week	MH	CO_2	164RC1		*Staphylococcus chromogenes*	+	-	-	-	-	-	-	-	-
ES1	-1 week	MH	CO_2	165RC1		*Staphylococcus hyicus*	+	-	-	-	-	-	-	-	-
ES1	-1 week	MH	CO_2	166RC1	yes	*Staphylococcus hyicus*	+	-	-	-	-	-	-	-	-
ES1	-1 week	MH	CO_2	167RC1		*Staphylococcus hyicus*	+	-	-	-	-	-	-	-	-
ES1	-1 week	MH	CO_2	168RC1		*Streptococcus gallolyticus/macedonicus*	+	-	-	-	-	-	-	-	-
ES1	-1 week	MH	CO_2	169RC1		*Streptococcus gallolyticus*	+	-	-	-	-	-	-	-	-
ES1	-1 week	MH	CO_2	170RC1		*Streptococcus gallolyticus/macedonicus*	+	-	-	-	-	-	-	-	-
ES1	-1 week	MH	CO_2	171RC1		*Streptococcus gallolyticus/macedonicus*	+	-	-	-	+	-	-	-	-
ES1	-1 week	MRS	CO_2	172RC1		*Lactobacillus salivarius*	+	-	-	-	+	-	-	-	-
ES1	-1 week	MRS	CO_2	173RC1		*Streptococcus gallolyticus/macedonicus*	+	-	-	-	-	-	-	-	-
ES1	-1 week	MRS	CO_2	174RC1		*Streptococcus gallolyticus/macedonicus*	+	-	-	-	-	-	-	-	-
ES1	-1 week	MRS	CO_2	175RC1		*Streptococcus gallolyticus/macedonicus*	+	-	-	-	-	-	-	-	-
ES1	-1 week	MRS	CO_2	176RC1		*Streptococcus gallolyticus/macedonicus*	+	-	-	-	-	-	-	-	-
ES1	-1 week	MC	CO_2	177RC1		*Escherichia coli*	-	-	-	-	-	-	-	-	-
ES1	-1 week	MC	CO_2	178RC1		*Escherichia coli*	-	-	-	-	-	-	-	-	-
ES1	-1 week	MC	CO_2	179RC1		*Escherichia coli*	-	-	-	-	-	-	-	-	-
ES1	-1 week	MC	CO_2	180RC1		*Escherichia coli*	-	-	-	-	-	-	-	-	-
ES1	-1 week	MC	CO_2	181RC1		*Escherichia coli*	-	-	-	-	-	-	-	-	-
ES1	-1 week	MC	CO_2	182RC1		*Escherichia coli*	-	-	-	-	-	-	-	-	-
ES1	-1 week	MC	CO_2	183RC1		*Pasteurella aerogenes*	-	-	-	-	-	-	-	-	-
ES1	-1 week	MC	CO_2	184RC1		*Pasteurella aerogenes*	-	-	-	-	-	-	-	-	-
ES1	-1 week	THB	CO_2	185RC1		*Staphylococcus chromogenes*	+	-	-	-	-	-	-	-	-
ES1	-1 week	THB	CO_2	186RC1		*Staphylococcus chromogenes*	+	-	-	-	-	-	-	-	-
ES1	-1 week	THB	CO_2	187RC1		*Staphylococcus hyicus*	+	-	-	-	-	-	-	-	-
ES1	-1 week	THB	CO_2	188RC1	yes	*Staphylococcus hyicus*	+	-	-	-	-	-	-	-	-

Supplementary Table 1. Continued.

								Inhibition assays									
Farm	Pig age	Medium	Atm	ID	WGS	16S ID	Gram	*Streptococcus suis J28*	*Streptococcus suis S10*	*Streptococcus suis P1/7*	*Streptococcus porci DSM 23759*	*Streptococcus porcinus DSM 20725*	*Streptococcus parasuis DSM 29126*	*Staphylococcus aureus L100*	*Escherichia coli L4242*	*Pasteurella aerogenes DMS 21448*	*Moraxella pluranimalium DSM 22804*
ES1	-1 week	THB	CO_2	189RC1		*Staphylococcus hyicus*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	THB	CO_2	190RC1		*Staphylococcus hyicus*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	THB	CO_2	191RC1		*Streptococcus acidominimus*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	THB	CO_2	192RC1		*Streptococcus suis*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	THB	CO_2	193RC1		*Streptococcus suis*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	THB	CO_2	194RC1		*Streptococcus suis*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	THB	CO_2	195RC1		*Rothia nasisuis*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	THB	CO_2	196RC1	yes	*Rothia nasisuis*	+	+	+/-	+	+	+	+	-	-	-	-
ES1	-1 week	THB	CO_2	197RC1	yes	*Staphylococcus hyicus*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	THB	CO_2	198RC1	yes	*Streptococcus suis*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	THB	CO_2	199RC1		*Staphylococcus hyicus*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	THB	CO_2	200RC1		*Streptococcus suis*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	THB	CO_2	201RC1		*Streptococcus suis*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	M17	CO_2	202RC1		*Streptococcus suis*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	M17	CO_2	203RC1		*Streptococcus gallolyticus/macedonicus*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	M17	CO_2	204RC1		*Streptococcus gallolyticus/macedonicus*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	M17	CO_2	205RC1		*Streptococcus gallolyticus/macedonicus*	+	-	-	-	-	+	-	-	-	-	-
ES1	-1 week	M17	CO_2	206RC1	yes	*Rothia nasimurium*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	M17	CO_2	207RC1	yes	*Rothia nasimurium*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	M17	CO_2	208RC1		*Staphylococcus hyicus*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	M17	CO_2	209RC1	yes	*Corynebacterium kozikiae*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	M17	CO_2	210RC1		*Streptococcus suis*	+	-	-	-	-	-	-	-	-	-	-

ES1	-1 week	M17	CO_2	211RC1		*Streptococcus suis*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	M17	CO_2	212RC1		*Staphylococcus hyicus*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	M17	CO_2	213RC1	yes	*Rothia nasimurium*	+	-	-	-	-	-	-	-	-	-	-
ES1	-1 week	M17	CO_2	214RC1		*Staphylococcus hyicus*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	THB	CO_2	1QC2CO		*Staphylococcus sciuri*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	THB	CO_2	2QC2CO		*Staphylococcus sciuri*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	THB	CO_2	3QC2CO		*Aerococcus urinaeequi*	+	-	-	-	-	-	-	+	-	-	-
ES2	-1 week	THB	CO_2	4QC2CO		*Aerococcus urinaeequi*	+	-	-	-	-	-	-	+	-	-	-
ES2	-1 week	THB	CO_2	5QC2CO	yes	*Corynebacterium kozikiae*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	THB	CO_2	6QC2CO		*Streptococcus pluranimalium*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	THB	CO_2	7QC2CO		*Aerococcus urinaeequi*	+	-	-	-	-	-	-	+	-	-	-
ES2	-1 week	THB	CO_2	8QC2CO	yes	*Rothia nasimurium*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	THB	CO_2	9QC2CO		*Staphylococcus sciuri*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	THB	CO_2	10QC2CO		*Staphylococcus sciuri*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	THB	CO_2	11QC2CO		*Staphylococcus hominis*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	THB	CO_2	12QC2CO		*Staphylococcus xylosus*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	M17	CO_2	14QC2CO		*Corynebacterium phoceense*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	M17	CO_2	15QC2CO	yes	*Rothia nasimurium*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	M17	CO_2	16QC2CO		*Staphylococcus sciuri*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	M17	CO_2	17QC2CO		*Staphylococcus sciuri*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	M17	CO_2	18QC2CO		*Staphylococcus gallinarum*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	M17	CO_2	19QC2CO		*Staphylococcus sciuri*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	M17	CO_2	20QC2CO		*Staphylococcus xylosus*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	M17	CO_2	21QC2CO		*Staphylococcus saprophyticus*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	M17	CO_2	22QC2CO	yes	*Rothia nasimurium*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	M17	CO_2	23QC2CO	yes	*Streptococcus suis*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	M17	CO_2	24QC2CO		*Corynebacterium phoceense*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	M17	CO_2	25QC2CO		*Streptococcus suis*	+	+	-	+	+	-	+	-	-	-	-
ES2	-1 week	M17	CO_2	26QC2CO		*Streptococcus suis*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	BHI	CO_2	27QC2CO		*Pediococcus pentosaceus*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	BHI	CO_2	28QC2CO	yes	*Rothia nasimurium*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	BHI	CO_2	29QC2CO		*Corynebacterium phoceense*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	BHI	CO_2	30QC2CO		*Staphylococcus sciuri*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	BHI	CO_2	31QC2CO		*Staphylococcus sciuri*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	BHI	CO_2	32QC2CO	yes	*Staphylococcus gallinarum*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	BHI	CO_2	33QC2CO		*Staphylococcus sciuri*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	BHI	CO_2	34QC2CO		*Rothia nasimurium*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	BHI	CO_2	35QC2CO		*Corynebacterium phoceense*	+	-	-	-	-	-	-	-	-	-	-

Supplementary Table 1. Continued.

								Inhibition assays										
Farm	Pig age	Medium	Atm	ID	WGS	16S ID	Gram	*Streptococcus suis J28*	*Streptococcus suis S10*	*Streptococcus suis P1/7*	*Streptococcus porci DSM 23759*	*Streptococcus porcinus DSM 20725*	*Streptococcus parasuis DSM 29126*	*Staphylococcus aureus L100*	*Escherichia coli L4242*	*Pasteurella aerogenes DMS 21448*	*Moraxella pluranimalium DSM 22804*
ES2	-1 week	BHI	CO_2	36QC2CO		*Staphylococcus saprophyticus*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	BHI	CO_2	37QC2CO		*Staphylococcus saprophyticus*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	BHI	CO_2	38QC2CO	yes	*Rothia nasimurium*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	BHI	CO_2	39QC2CO		*Weissella paramesenteroides*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	BHI	CO_2	40QC2CO		*Streptococcus parasanguinis*	+	+/-	-	-	-	-	-	-	-	-	-
ES2	-1 week	MH	CO_2	41QC2CO		*Staphylococcus sciuri*	+	+/-	-	-	-	-	-	-	-	-	-
ES2	-1 week	MH	CO_2	42QC2CO		*Staphylococcus sciuri*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	MH	CO_2	44QC2CO		*Staphylococcus sciuri*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	MH	CO_2	43QC2CO		*Escherichia coli*	-	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	MH	CO_2	45QC2CO		*Streptococcus suis*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	MH	CO_2	47QC2CO		*Staphylococcus sciuri*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	MH	CO_2	48QC2CO	yes	*Rothia nasimurium*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	MH	CO_2	50QC2CO		*Staphylococcus sciuri*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	MH	CO_2	51QC2CO		*Staphylococcus xylosus*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	MH	CO_2	52QC2CO		*Staphylococcus xylosus*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	MH	CO_2	53QC2CO		*Corynebacterium amycolatum*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	MH	CO_2	54QC2CO	yes	*Rothia nasimurium*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	LB	CO_2	55QC2CO		*Staphylococcus saprophyticus*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	LB	CO_2	56QC2CO		*Staphylococcus sciuri*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	LB	CO_2	57QC2CO		*Staphylococcus saprophyticus*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	LB	CO_2	58QC2CO		*Staphylococcus sciuri*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	LB	CO_2	59QC2CO		*Streptococcus suis*	+	-	-	-	-	-	-	-	-	-	-

ES2	-1 week	LB	CO_2	60QC2CO	yes	*Corynebacterium kozikiae*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	LB	CO_2	61QC2CO		*Corynebacterium amycolatum*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	LB	CO_2	62QC2CO		*Staphylococcus cohnii*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	SBA	CO_2	63QC2CO	yes	*Rothia nasimurium*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	SBA	CO_2	64QC2CO	yes	*Rothia nasimurium*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	SBA	CO_2	66QC2CO		*Rothia nasimurium*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	SBA	CO_2	67QC2CO		*Rothia nasimurium*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	SBA	CO_2	68QC2CO	yes	*Brevibacterium*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	SBA	CO_2	69QC2CO		*Staphylococcus sciuri*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	SBA	CO_2	70QC2CO		*Streptococcus suis*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	SBA	CO_2	71QC2CO		*Streptococcus acidominimus*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	SBA	CO_2	72QC2CO		*Lactobacillus johnsonii*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	SBA	CO_2	73QC2CO		*Corynebacterium phoceense*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	SBA	CO_2	74QC2CO		*Streptococcus suis*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	SBA	CO_2	75QC2CO		*Neisseria wadsworthii*	-	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	SBA	CO_2	76QC2CO	yes	*Corynebacterium kozikiae*	+	++	-	++	++	-	++	+	-	+/-	-
ES2	-1 week	SBA	CO_2	77QC2CO		*Corynebacterium amycolatum*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	SBA	CO_2	78QC2CO		*Streptococcus suis*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	MC	CO_2	79QC2CO		*Escherichia coli*	-	-	-	-	-	-	-	-	++	-	-
ES2	-1 week	MC	CO_2	82QC2CO		*Ochrobactrum anthropi*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	MC	CO_2	85QC2CO		*Escherichia coli*	-	-	-	-	-	-	-	-	++	-	-
ES2	-1 week	MC	CO_2	89QC2CO		*Pasteurella aerogenes*	-	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	MRS	CO_2	91QC2CO		*Lactobacillus plantarum*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	MRS	CO_2	92QC2CO		*Lactobacillus plantarum*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	MRS	CO_2	93QC2CO		*Pediococcus pentosaceus*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	MRS	CO_2	94QC2CO		*Pediococcus pentosaceus*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	MRS	CO_2	97QC2CO		*Pediococcus pentosaceus*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	MRS	CO_2	98QC2CO		*Pediococcus pentosaceus*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	MRS	CO_2	99QC2CO		*Pediococcus pentosaceus*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	MRS	CO_2	100QC2CO		*Escherichia coli S88*	-	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	MRS	CO_2	101QC2CO	yes	*Corynebacterium phoceense*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	MRS	CO_2	103QC2CO		*Corynebacterium kozikiae*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	MRS	CO_2	104QC2CO		*Rothia nasimurium*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	THB	O_2	1QC2O2		*Rothia nasimurium*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	THB	O_2	2QC2O2	yes	*Rothia nasimurium*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	THB	O_2	3QC2O2	yes	*Rothia nasimurium*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	THB	O_2	4QC2O2	yes	*Rothia nasimurium*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	THB	O_2	6QC2O2	yes	*Rothia nasimurium*	+	-	-	-	-	-	-	-	-	-	-

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Supplementary Table 1. Continued.

								Inhibition assays									
Farm	Pig age	Medium	Atm	ID	WGS	16S ID	Gram	*Streptococcus suis J28*	*Streptococcus suis S10*	*Streptococcus suis P1/7*	*Streptococcus porci DSM 23759*	*Streptococcus porcinus DSM 20725*	*Streptococcus parasuis DSM 29126*	*Staphylococcus aureus L100*	*Escherichia coli L4242*	*Pasteurella aerogenes DMS 21448*	*Moraxella pluranimalium DSM 22804*
ES2	-1 week	THB	O_2	7QC2O2	yes	*Rothia nasimurium*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	THB	O_2	8QC2O2	yes	*Staphylococcus sciuri*	+	-	-	-	-	-	-	-	-	+	+
ES2	-1 week	THB	O_2	9QC2O2		*Corynebacterium xerosis*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	THB	O_2	10QC2O2		*Staphylococcus sciuri*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	THB	O_2	11QC2O2		*Corynebacterium phoceense*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	THB	O_2	12QC2O2		*Staphylococcus saprophyticus*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	THB	O_2	13QC2O2		*Staphylococcus xylosus*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	THB	O_2	14QC2O2		*Moraxella porci*	-	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	THB	O_2	15QC2O2		*Aerococcus urinaeequi*	+	-	-	-	-	-	-	+	-	-	-
ES2	-1 week	THB	O_2	16QC2O2		*Streptococcus suis*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	THB	O_2	17QC2O2		*Streptococcus porcorum*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	THB	O_2	18QC2O2		*Corynebacterium xerosis*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	M17	O_2	19QC2O2	yes	*Rothia nasimurium*	+	-	-	-	-	-	-	-	-	+	-
ES2	-1 week	M17	O_2	20QC2O2	yes	*Corynebacterium phoceense*	+	-	-	-	-	-	-	-	-	+/-	+/-
ES2	-1 week	M17	O_2	21QC2O2		*Streptococcus suis*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	M17	O_2	22QC2O2		*Staphylococcus sciuri*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	M17	O_2	23QC2O2		*Staphylococcus sciuri*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	M17	O_2	24QC2O2		*Staphylococcus xylosus*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	M17	O_2	25QC2O2		*Staphylococcus xylosus*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	M17	O_2	26QC2O2		*Corynebacterium kozikiae*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	M17	O_2	27QC2O2		*Streptococcus suis*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	M17	O_2	28QC2O2		*Streptococcus suis*	+	-	-	+	+	-	+	-	-	-	-

ES2	-1 week	M17	O_2	29QC2O2		*Staphylococcus hominis*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	M17	O_2	30QC2O2		*Corynebacterium phoceense*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	M17	O_2	32QC2O2		*Staphylococcus gallinarum*	+	-	-	-	-	-	-	-	-	-	+
ES2	-1 week	BHI	O_2	33QC2O2		*Lactobacillus zhachilii*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	BHI	O_2	34QC2O2	yes	*Rothia nasimurium*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	BHI	O_2	35QC2O2		*Streptococcus suis*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	BHI	O_2	36QC2O2	yes	*Rothia nasimurium*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	BHI	O_2	37QC2O2	yes	*Rothia nasimurium*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	BHI	O_2	38QC2O2		*Corynebacterium phoceense*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	BHI	O_2	39QC2O2		*Moraxella porci*	-	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	BHI	O_2	40QC2O2		*Staphylococcus saprophyticus*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	BHI	O_2	41QC2O2		*Aerococcus urinaeequi*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	BHI	O_2	42QC2O2		*Streptococcus porcorum*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	BHI	O_2	46QC2O2	yes	*Rothia nasimurium*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	BHI	O_2	47QC2O2		*Staphylococcus sciuri*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	BHI	O_2	48QC2O2		*Shingobacterium mizutaii*	-	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	BHI	O_2	49QC2O2		*Weissella paramesenteroides*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	MH	O_2	50QC2O2	yes	*Brevibacterium*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	MH	O_2	51QC2O2		*Corynebacterium xerosis*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	MH	O_2	52QC2O2		*Staphylococcus saprophyticus*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	MH	O_2	53QC2O2		*Enterococcus italicus*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	MH	O_2	54QC2O2		*Staphylococcus gallinarum*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	MH	O_2	55QC2O2		*Corynebacterium kozikiae*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	MH	O_2	56QC2O2	yes	*Rothia endophytica*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	MH	O_2	57QC2O2		*Rothia nasimurium*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	MH	O_2	58QC2O2		*Staphylococcus sciuri*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	MH	O_2	59QC2O2		*Corynebacterium kozikiae*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	MH	O_2	60QC2O2		*Corynebacterium phoceense*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	MH	O_2	61QC2O2		*Rothia nasimurium*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	MH	O_2	62QC2O2		*Rothia nasimurium*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	LB	O_2	63QC2O2	yes	*Staphylococcus borealis*	+	-	-	-	-	-	-	-	-	-	+
ES2	-1 week	LB	O_2	64QC2O2		*Rothia nasimurium*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	LB	O_2	67QC2O2		*Corynebacterium phoceense*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	LB	O_2	68QC2O2		*Corynebacterium phoceense*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	LB	O_2	69QC2O2		*Staphylococcus sciuri*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	LB	O_2	70QC2O2	yes	*Corynebacterium phoceense*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	LB	O_2	71QC2O2		*Staphylococcus saprophyticus*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	LB	O_2	72QC2O2	yes	*Staphylococcus cohnii*	+	-	-	-	-	-	-	-	-	-	-

Supplementary Table 1. Continued.

								Inhibition assays									
Farm	Pig age	Medium	Atm	ID	WGS	16S ID	Gram	*Streptococcus suis J28*	*Streptococcus suis S10*	*Streptococcus suis P1/7*	*Streptococcus porci DSM 23759*	*Streptococcus porcinus DSM 20725*	*Streptococcus parasuis DSM 29126*	*Staphylococcus aureus L100*	*Escherichia coli L4242*	*Pasteurella aerogenes DMS 21448*	*Moraxella pluranimalium DSM 22804*
ES2	-1 week	LB	O_2	73QC2O2		*Staphylococcus cohnii*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	LB	O_2	74QC2O2		*Rothia nasimurium*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	LB	O_2	76QC2O2		*Staphylococcus saprophyticus*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	SBA	O_2	77QC2O2		*Staphylococcus gallinarum*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	SBA	O_2	78QC2O2		*Weissella paramesenteroides*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	SBA	O_2	79QC2O2		*Staphylococcus saprophyticus*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	SBA	O_2	80QC2O2		*Brachybacterium paraconglomeratum*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	SBA	O_2	81QC2O2		*Weissella paramesenteroides*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	SBA	O_2	82QC2O2		*Corynebacterium kozikiae*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	SBA	O_2	83QC2O2		*Staphylococcus xylosus*	+	+/-	-	-	-	-	+	-	-	-	-
ES2	-1 week	SBA	O_2	84QC2O2		*Weissella paramesenteroides*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	SBA	O_2	85QC2O2		*Rothia nasimurium*	+	+/-	-	-	-	-	-	-	-	-	-
ES2	-1 week	SBA	O_2	86QC2O2		*Streptococcus suis*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	SBA	O_2	88QC2O2		*Staphylococcus sciuri*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	SBA	O_2	89QC2O2		*Pasteurella aerogenes*	-	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	SBA	O_2	90QC2O2		*Aerococcus viridans*	+	-	-	-	-	-	-	++	+	-	-
ES2	-1 week	SBA	O_2	91QC2O2	yes	*Brevibacterium*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	SBA	O_2	92QC2O2	yes	*Corynebacterium phoceense*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	SBA	O_2	93QC2O2		*Lactococcus lactis*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	SBA	O_2	94QC2O2		*Corynebacterium kozikiae*	+	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	MC	O_2	95QC2O2		*Serratia rubidaea*	-	-	-	-	-	-	-	-	+	-	-
ES2	-1 week	MC	O_2	96QC2O2		*Escherichia coli*	-	-	-	-	-	-	-	-	-	-	-

ES2	-1 week	MC	O_2	97QC2O2		*Escherichia coli*	-	-	-	-	-	-	-	++	-	-
ES2	-1 week	MC	O_2	99QC2O2		*Shigella flexneri*	-	-	-	-	-	-	-	+	-	-
ES2	-1 week	MC	O_2	101QC2O2		*Staphylococcus chromogenes*	+	-	-	-	-	-	-	-	-	-
ES2	-1 week	MC	O_2	103QC2O2		*Escherichia coli*	-	-	-	-	-	-	-	-	-	-
ES2	-1 week	MRS	O_2	104QC2O2		*Lactobacillus plantarum*	+	-	-	-	-	-	-	-	-	-
ES2	-1 week	MRS	O_2	105QC2O2	yes	*Pediococcus pentosaceus*	+	-	-	-	-	-	-	-	+	-
ES2	-1 week	MRS	O_2	109QC2O2		*Lactobacillus plantarum*	+	-	-	-	-	-	-	-	-	-
ES2	-1 week	MRS	O_2	111QC2O2	yes	*Pediococcus pentosaceus*	+	-	-	-	-	-	-	-	+	-
ES2	-1 week	MRS	O_2	112QC2O2		*Lactobacillus plantarum*	+	-	-	-	-	-	-	-	-	-
ES2	week +3	CA	O_2	1QC3O2		*Rothia nasimurium*	+	-	-	-	-	-	-	-	-	-
ES2	week +3	CA	O_2	2QC3O2		*Rothia nasimurium*	+	-	-	-	-	-	-	-	-	-
ES2	week +3	CA	O_2	3QC3O2		*Escherichia coli*	+	-	-	-	-	-	-	-	-	-
ES2	week +3	CA	O_2	8QC3O2		*Rothia nasimurium*	+	-	-	-	+/-	-	-	-	-	-
ES2	week +3	CA	O_2	9QC3O2		*Rothia nasimurium*	+	-	-	-	-	-	-	-	-	-
ES2	week +3	CA	O_2	10QC3O2		*Rothia nasimurium*	+	+/-	-	-	-	-	-	-	-	-
ES2	week +3	CA	O_2	12QC3O2		*Streptococcus acidominimus*	+	+/-	-	-	+/-	-	-	-	-	-
ES2	week +3	CA	O_2	13QC3O2		*Rothia nasimurium*	+	-	-	-	-	-	-	-	-	-
ES2	week +3	CA	O_2	14QC3O2		*Rothia nasimurium*	+	-	-	-	-	-	-	-	-	-
ES2	week +3	MC	O_2	21QC3O2		*Escherichia coli*	-	-	-	-	-	-	-	-	-	-
ES2	week +3	MC	O_2	22QC3O2		*Escherichia coli*	-	-	-	-	-	-	-	-	-	-
ES2	week +3	BHI	O_2	23QC3O2		*Escherichia coli*	-	-	-	-	-	-	-	-	-	-
ES2	week +3	BHI	O_2	24QC3O2		*Staphylococcus aureus*	+	-	-	-	-	-	-	-	-	-
ES2	week +3	BHI	O_2	25QC3O2		*Staphylococcus aureus*	+	-	-	-	-	-	-	-	-	-
ES2	week +3	BHI	O_2	26QC3O2		*Staphylococcus microti*	+	-	-	-	+/-	-	-	-	-	-
ES2	week +3	BHI	O_2	27QC3O2		*Rothia nasimurium*	+	-	-	-	-	-	-	-	-	-
ES2	week +3	BHI	O_2	28QC3O2		*Staphylococcus aureus*	+	-	-	-	-	-	-	-	-	-
ES2	week +3	BHI	O_2	29QC3O2		*Staphylococcus chromogenes*	+	-	-	-	-	-	-	-	-	-
ES2	week +3	BHI	O_2	30QC3O2		*Rothia nasimurium*	+	-	-	-	-	-	-	-	-	-
ES2	week +3	BHI	O_2	31QC3O2		*Staphylococcus aureus*	+	-	-	-	-	-	-	-	-	-
ES2	week +3	BHI	O_2	32QC3O2		*Rothia nasimurium*	+	-	-	-	-	-	-	-	-	-
ES2	week +3	BHI	O_2	33QC3O2		*Streptococcus porcorum*	+	-	-	-	-	-	-	-	+/-	-
ES2	week +3	CA	CO_2	1QC3CO		*Rothia nasimurium*	+	-	-	-	-	-	-	-	-	-
ES2	week +3	CA	CO_2	2QC3CO		*Rothia nasimurium*	+	-	-	-	-	-	-	-	-	-
ES2	week +3	CA	CO_2	3QC3CO		*Rothia nasimurium*	+	-	-	-	-	-	-	-	-	-
ES2	week +3	CA	CO_2	4QC3CO		*Aerococcus viridans*	+	-	-	-	-	-	-	+	-	-
ES2	week +3	CA	CO_2	5QC3CO		*Streptococcus porcorum*	+	-	-	-	-	-	-	-	-	-
ES2	week +3	CA	CO_2	6QC3CO		*Streptococcus suis*	+	-	-	-	-	-	-	-	-	-
ES2	week +3	CA	CO_2	7QC3CO		*Staphylococcus aureus*	+	-	-	-	-	-	-	-	-	-

Supplementary Table 1. Continued.

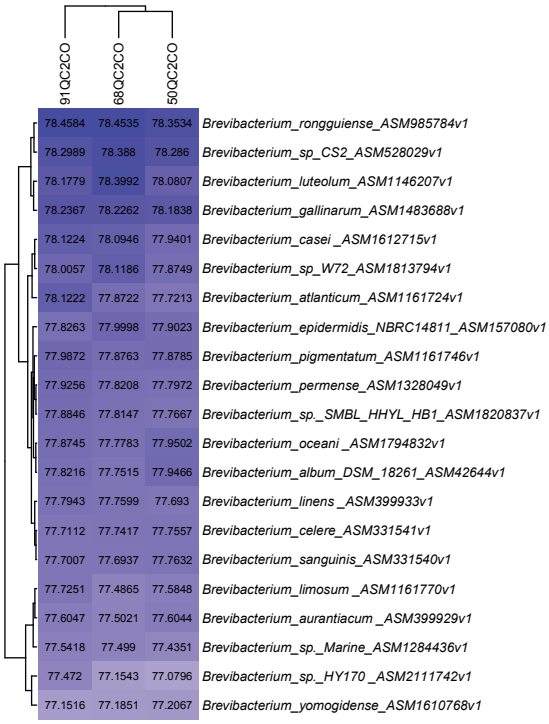
								Inhibition assays										
Farm	Pig age	Medium	Atm	ID	WGS	16S ID	Gram	*Streptococcus suis J28*	*Streptococcus suis S10*	*Streptococcus suis P1/7*	*Streptococcus porci DSM 23759*	*Streptococcus porcinus DSM 20725*	*Streptococcus parasuis DSM 29126*	*Staphylococcus aureus L100*	*Escherichia coli L4242*	*Pasteurella aerogenes DMS 21448*	*Moraxella pluranimalium DSM 22804*
ES2	week +3	CA	CO_2	8QC3CO		*Streptococcus porcorum*	+	-	-	-	-	-	-	-	-	-	-
ES2	week +3	CA	CO_2	12QC3CO		*Escherichia coli*	-	-	-	-	-	-	-	-	-	-	-
ES2	week +3	CA	CO_2	13QC3CO		*Rothia nasimurium*	+	-	-	-	-	-	-	-	-	-	-
ES2	week +3	SA	CO_2	16QC3CO		*Shigella sonnei*	-	-	-	-	-	-	-	-	+	-	-
ES2	week +3	SA	CO_2	17QC3CO		*Escherichia coli*	-	-	-	-	-	-	-	-	-	-	-
ES2	week +3	MC	CO_2	21QC3CO		*Shigella sonnei*	-	-	-	-	-	-	-	-	+	-	-
ES2	week +3	BHI	CO_2	22QC3CO		*Rothia nasimurium*	+	-	-	-	-	-	-	-	-	-	-
ES2	week +3	BHI	CO_2	23QC3CO		*Rothia nasimurium*	+	-	-	-	-	-	-	-	-	-	-
ES2	week +3	BHI	CO_2	24QC3CO		*Rothia nasimurium*	+	-	-	-	-	-	-	-	-	-	-
ES2	week +3	BHI	CO_2	25QC3CO		*Rothia nasimurium*	+	-	-	-	-	-	-	-	-	-	-
ES2	week +3	BHI	CO_2	26QC3CO		*Escherichia coli*	-	-	-	-	-	-	-	-	-	-	-
ES2	week +3	BHI	CO_2	27QC3CO		*Staphylococcus chromogenes*	+	-	-	-	-	-	-	-	-	-	-
ES2	week +3	BHI	CO_2	28QC3CO		*Streptococcus porcorum*	+	-	-	-	-	-	-	-	-	-	-
ES2	week +3	BHI	CO_2	29QC3CO		*Streptococcus porcorum*	+	-	-	-	-	-	-	-	-	-	-
ES2	week +3	MC	O_2	1QC4O2		*Escherichia coli*	-	-	-	-	-	-	-	-	-	-	-
ES2	week +3	MC	O_2	2QC4O2		*Escherichia coli*	-	-	-	-	-	-	-	-	-	-	-
ES2	week +3	MC	O_2	3QC4O2		*Shigella sonnei*	-	-	-	-	-	-	-	-	-	-	-
ES2	week +3	BHI	O_2	4QC4O2	yes	*Corynebacterium phoceense*	+	-	-	-	-	-	-	-	-	-	-
ES2	week +3	BHI	O_2	5QC4O2		*Staphylococcus argenteus*	+	-	-	-	-	-	-	-	-	-	-
ES2	week +3	BHI	O_2	6QC4O2		*Rothia nasimurium*	+	-	-	-	-	-	-	-	-	-	-
ES2	week +3	BHI	O_2	7QC4O2		*Rothia nasimurium*	+	-	-	-	-	-	-	-	-	-	-
ES2	week +3	BHI	O_2	8QC4O2		*Staphylococcus aureus*	+	-	-	-	-	-	-	-	-	-	-

ES2	week +3	BHI	O_2	9QC4O2		*Staphylococcus aureus*	+	-	-	-	-	-	-	-
ES2	week +3	BHI	O_2	10QC4O2		*Rothia nasimurium*	+	-	-	-	-	-	-	-
ES2	week +3	CA	O_2	11QC4O2		*Rothia nasimurium*	+	-	-	-	-	-	-	-
ES2	week +3	CA	O_2	13QC4O2		*Rothia nasimurium*	+	-	-	-	-	-	-	-
ES2	week +3	CA	O_2	14QC4O2		*Rothia nasimurium*	+	-	-	-	-	-	-	-
ES2	week +3	CA	O_2	15QC4O2	yes	*Rothia nasimurium*	+	-	-	-	-	-	+	-
ES2	week +3	CA	O_2	16QC4O2		*Staphylococcus aureus*	+	-	-	-	-	-	-	-
ES2	week +3	CA	O_2	17QC4O2		*Rothia nasimurium*	+	-	-	-	-	-	-	-
ES2	week +3	CA	O_2	18QC4O2	yes	*Rothia nasimurium*	+	-	-	-	-	-	+	-
ES2	week +3	CA	O_2	19QC4O2		*Staphylococcus aureus*	+	-	-	-	-	-	-	-
ES2	week +3	CA	O_2	20QC4O2		*Streptococcus suis*	+	-	-	-	-	-	-	-
ES2	week +3	BHI	O_2	21QC4O2		*Escherichia coli*	-	-	-	-	-	-	+	-
ES2	week +3	BHI	O_2	22QC4O2		*Escherichia coli*	-	-	-	-	-	-	+	-
ES2	week +3	BHI	O_2	23QC4O2		*Escherichia coli*	-	-	-	-	-	-	-	-
ES2	week +3	BHI	O_2	24QC4O2		*Escherichia coli*	-	-	-	-	-	-	-	-
ES2	week +3	BHI	O_2	25QC4O2		*Escherichia coli*	-	-	-	-	-	-	-	-
ES2	week +3	BHI	O_2	26QC4O2		*Escherichia coli*	-	-	-	-	-	-	-	-
ES2	week +3	BHI	O_2	27QC4O2		*Escherichia coli*	-	-	-	-	-	-	-	-
ES2	week +3	BHI	O_2	28QC4O2		*Escherichia coli*	-	-	-	-	-	-	-	-
ES2	week +3	BHI	CO_2	1QC4CO		*Escherichia coli*	-	-	-	-	-	-	-	-
ES2	week +3	BHI	CO_2	2QC4CO		*Staphylococcus argenteus*	+	-	-	-	-	-	-	-
ES2	week +3	BHI	CO_2	4QC4CO		*Streptococcus suis*	+	-	-	-	-	-	-	-
ES2	week +3	BHI	CO_2	6QC4CO		*Escherichia coli*	-	-	-	-	-	-	-	-
ES2	week +3	BHI	CO_2	7QC4CO		*Escherichia coli*	-	-	-	-	-	-	-	-
ES2	week +3	BHI	CO_2	8QC4CO		*Escherichia coli*	-	-	-	-	-	-	-	-
ES2	week +3	BHI	CO_2	9QC4CO		*Escherichia coli*	-	-	-	-	-	-	-	-
ES2	week +3	BHI	CO_2	10QC4CO		*Escherichia coli*	-	-	-	-	-	-	-	-
ES2	week +3	CA	CO_2	11QC4CO		*Enterococcus villorum*	+	-	-	-	-	-	-	-
ES2	week +3	CA	CO_2	12QC4CO		*Rothia nasimurium*	+	-	-	-	-	-	-	-
ES2	week +3	CA	CO_2	13QC4CO		*Rothia nasimurium*	+	-	-	-	-	-	-	-
ES2	week +3	CA	CO_2	14QC4CO		*Moraxella pluranimalium*	-	-	-	-	-	-	-	-
ES2	week +3	CA	CO_2	15QC4CO		*Staphylococcus hyicus*	+	-	-	-	-	-	-	-
ES2	week +3	MC	CO_2	16QC4CO		*Escherichia coli*	-	-	-	-	-	-	-	-
ES2	week +3	MC	CO_2	17QC4CO		*Escherichia coli*	-	-	-	-	-	-	-	-
ES2	week +3	BHI	CO_2	18QC4CO		*Staphylococcus aureus*	+	-	-	-	-	-	-	-
ES2	week +3	BHI	CO_2	19QC4CO		*Rothia nasimurium*	+	-	-	-	-	-	-	-
ES2	week +3	BHI	CO_2	20QC4CO		*Rothia nasimurium*	+	-	-	-	-	-	-	-
ES2	week +3	BHI	CO_2	21QC4CO		*Streptococcus suis*	+	-	-	-	-	-	-	-

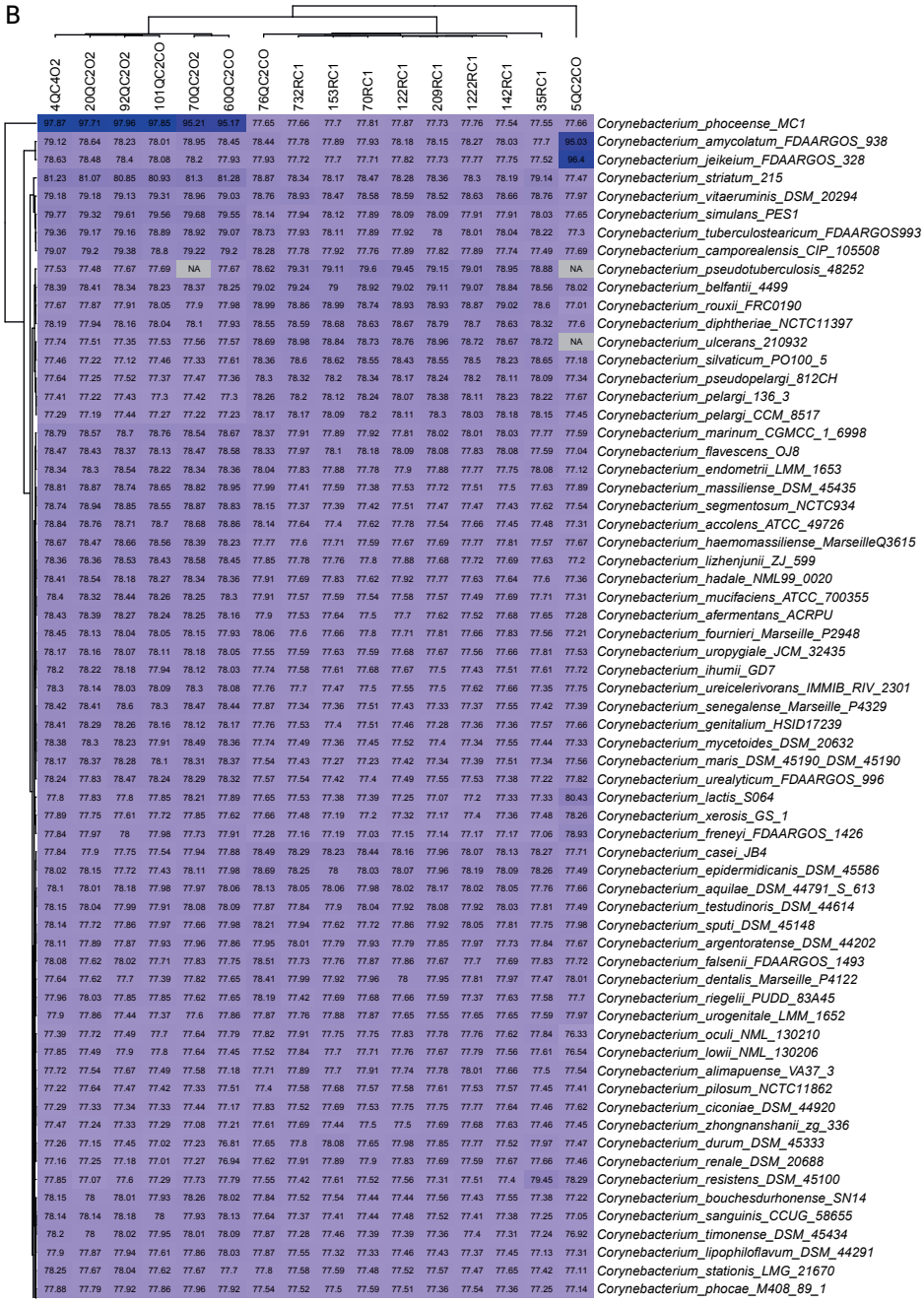
Supplementary Table 1. Continued.

								Inhibition assays										
Farm	Pig age	Medium	Atm	ID	WGS	16S ID	Gram	*Streptococcus suis J28*	*Streptococcus suis S10*	*Streptococcus suis P1/7*	*Streptococcus porci DSM 23759*	*Streptococcus porcinus DSM 20725*	*Streptococcus parasuis DSM 29126*	*Staphylococcus aureus L100*	*Escherichia coli L4242*	*Pasteurella aerogenes DMS 21448*	*Moraxella pluranimalium DSM 22804*
ES2	week +3	BHI	CO_2	22QC4CO		*Escherichia coli*	-	-	-	-	-	-	-	-	-	-	-
NL	week +7	MC	AN	1QC2AN		*Escherichia coli*	-	-	-	-	-	-	-	-	+	-	-
NL	week +7	MC	AN	2QC2AN		*Escherichia coli*	-	-	-	-	-	-	-	-	+	-	-
NL	week +7	MC	AN	3QC2AN		*Escherichia coli*	-	-	-	-	-	-	-	-	-	-	-
NL	week +7	MH	AN	4QC2AN		*Escherichia coli*	-	-	-	-	-	-	-	-	+	-	-
NL	week +7	MH	AN	5QC2AN		*Streptococcus porcinus*	+	+	-	+	+/-	-	+/-	-	-	-	-
NL	week +7	MH	AN	6QC3AN		*Escherichia coli*	-	-	-	-	-	-	-	-	-	-	-
NL	week +7	BHI	AN	7QC3AN		*Enterococcus faecalis*	+	-	-	-	+/-	-	+	-	-	-	-
NL	week +7	BHI	AN	8QC3AN		*Enterococcus hirae*	+	-	-	-	-	-	-	-	-	-	-
NL	week +7	BHI	AN	9QC3AN		*Enterococcus hirae*	+	-	-	-	-	-	-	-	-	-	-
NL	week +7	BHI	AN	10QC3AN		*Enterococcus hirae*	+	-	-	-	-	-	-	-	-	-	-
NL	week +7	BHI	AN	11QC3AN		*Streptococcus suis*	+	-	-	-	-	-	-	-	-	-	-
NL	week +7	BHI	AN	12QC3AN		*Streptococcus hyointestinalis*	+	+	+	+	-	+	-	-	-	-	-
NL	week +7	MRS	AN	13QC3AN		*Streptococcus gallolyticus*	+	+	+	+	-	+	+/-	-	-	-	-
NL	week +7	LB	AN	16QC3AN		*Escherichia coli*	-	-	-	-	-	-	-	-	+	-	-
NL	week +7	LB	AN	17QC3AN		*Enterococcus hirae*	+	-	-	-	-	-	-	-	-	-	-
NL	week +7	LB	AN	18QC3AN		*Escherichia coli*	-	-	-	-	-	-	-	-	-	-	-
NL	week +7	SBA	AN	19QC3AN		*Streptococcus dysgalactiae*	+	-	-	-	-	-	-	-	-	-	-
NL	week +7	MRS	AN	14QC3AN		*Lactobacillus johnsonii*	+	-	-	-	-	-	-	-	-	-	-
NL	week +7	MRS	AN	15QC3AN		*Lactobacillus johnsonii*	+	-	-	-	-	-	-	-	-	-	-
NL	week +7	SBA	AN	20QC3AN		*Lactobacillus johnsonii*	+	-	-	-	-	-	-	-	-	-	-
NL	week +7	SBA	AN	21QC3AN		*Fusobacterium necrophorum*	-	-	-	-	-	-	-	-	-	-	-

A



Supplementary Figure 2. The Average Nucleotide Identity (ANI) between the new proposed species of three *Brevibacterium moorei* **(A)** and the nine of the new proposed species *Corynebacterium kozikiae* **(B)** with RefSeq species.



Supplementary Figure 2. Continued.

NA	NA	NA	NA	NA	NA	77.4	77.72	77.64	77.37	77.32	77.33	77.44	77.36	77.27	77.24	*Corynebacterium_anserum_23H37_10*
78.23	77.78	78.12	78.27	78.21	78.3	77.34	77.52	77.26	77.33	77.35	77.32	77.42	77.26	77.43	77.16	*Corynebacterium_imitans_DSM_44264*
78.31	77.82	77.85	77.93	78	78.08	77.49	77.27	77.21	77.23	77.38	77.27	77.19	77.33	77.25	77.19	*Corynebacterium_qintianiae_MC1420*
77.78	77.98	78.01	78.05	77.83	77.88	77.15	77.27	77.35	77.39	77.38	77.26	77.26	77.16	77.2	77.37	*Corynebacterium_tapiri_LMG_28165*
77.57	77.58	77.75	77.66	77.72	77.39	77.4	77.37	77.54	77.25	77.36	77.39	77.51	77.36	77.27	77.62	*Corynebacterium_tuscaniense_UMB0792*
77.74	77.42	77.71	77.59	77.4	77.6	77.64	77.34	77.3	77.25	77.43	77.3	77.27	77.39	77.64	77.11	*Corynebacterium_lubricantis_DSM_45231*
77.94	77.93	77.79	77.44	78.16	77.67	77.93	77.36	77.32	77.18	77.22	77.31	77.17	76.97	76.9	77.05	*Corynebacterium_glucuronolyticum_FDAARGOS1053*
78.01	77.98	77.99	77.8	77.67	77.76	77.32	77.14	77.11	77.36	77.27	77.08	77.21	77.25	77.12	76.97	*Corynebacterium_uterequi_DSM_45634*
77.84	78.01	77.71	77.59	77.46	77.59	77.48	77.22	77.11	77.06	77.13	77.07	77.14	77.21	77.09	76.94	*Corynebacterium_pyruviciproducens_ATCC_BAA1742*
77.37	77.97	77.44	77.33	77.77	77.23	NA	76.79	NA	NA	NA	NA	NA	76.66	NA	77.69	*Corynebacterium_kroppenstedtii_FDAARGOS_1192*
77.41	77.38	77.41	77.52	77.53	77.59	77.37	77.47	77.07	77.04	77.17	77.01	77.31	77.12	77.11	76.89	*Corynebacterium_propinquum_FDAARGOS_1113*
77.56	77.61	77.69	77.49	77.3	77.69	77.01	77.04	77.01	76.91	77.02	77.16	77.11	77.1	77.12	76.91	*Corynebacterium_otitidis_ATCC_51513*
77.53	77.41	77.64	77.44	77.19	77.23	76.91	76.92	76.76	76.92	76.85	76.81	76.85	76.85	76.73	76.83	*Corynebacterium_capitovis_DSM_44611*
77.21	76.89	77.02	77.13	77.04	76.95	77.32	76.89	76.82	76.71	76.88	76.91	76.82	76.88	78.56	76.78	*Corynebacterium_atypicum_R2070*
77.55	77.08	77.47	77.09	77.11	76.79	78.03	77.22	77.34	77.31	77.16	77.17	77.17	77.03	77.05	77.88	*Corynebacterium_auriscanis_CIP_106629*
77.12	77.12	77.06	76.9	76.8	76.96	77.46	77.11	77.1	76.96	77.1	77.1	77.16	77	77.32	76.83	*Corynebacterium_canis_CCUG_58627*
76.95	77.02	76.96	77.03	76.44	76.75	77.35	76.88	77.01	77.02	77.2	77.06	77.04	77.06	76.91	77.33	*Corynebacterium_ulceribovis_DSM_45146*
NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	77.06	NA	77.01	76.79	NA	*Corynebacterium_freiburgense_DSM_45254*
77.24	77.04	77.08	77.19	77.26	77.2	76.88	76.83	76.83	76.87	77.05	76.97	77	76.66	77.12	77.25	*Corynebacterium_bovis_FDAARGOS_1052*
76.89	76.81	77.21	76.99	77.46	77.18	NA	76.83	NA	NA	NA	NA	76.81	76.83	76.67	77.44	*Corynebacterium_parakroppenstedtii_MC_26*

Supplementary Figure 2. Continued.

Supplementary Table 2. antiSMASH results across the genomes of the selected 45 isolates.

		WGS info				
		# contigs	Total length (bp)	GC (%)	N50	N75
ID	Assembly Accession					
Brevibacterium sp. 68QC2CO	JANIJX000000000	69	3556979	66,76	115056	84235
Brevibacterium sp. 91QC2O2	JANIJY000000000	63	3444434	66,93	143536	68949
Brevibacterium sp. 50QC2O2	JANIJZ000000000	57	3429606	66,91	165317	108327
Corynebacterium sp. 35RC1	JANIKA000000000	271	2475913	60,8	48099	26684
Corynebacterium sp. 70RC1	JANIKB000000000	72	2431450	60,5	80556	42221
Corynebacterium sp. 732RC1	JANIKC000000000	54	2387113	60,62	116172	65296
Corynebacterium sp. 122RC1	JANIKD000000000	57	2387684	60,62	95217	59915
Corynebacterium sp. 1222RC1	JANIKE000000000	62	2387559	60,62	96389	46382
Corynebacterium sp. 142RC1	JANIKF000000000	52	2430530	60,51	93234	70841
Corynebacterium sp. 153RC1	JANIKG000000000	54	2387249	60,62	137563	65314
Corynebacterium sp. 209RC1	JANIKH000000000	76	2397971	60,52	96389	48742
Corynebacterium sp. 5QC2CO	JANIKI000000000	31	2429181	58,91	197730	96313
Corynebacterium sp. 76QC2CO	JANIKJ000000000	57	2452417	60,36	123845	68177
Corynebacterium phoceense 20QC2O2	JANIKK000000000	72	2827415	63,34	125802	77130
Corynebacterium phoceense 101QC2CO	JANIKL000000000	46	2743271	63,52	196238	99883
Corynebacterium phoceense 4QC4O2	JANIKM000000000	56	2790166	63,57	124148	72424
Corynebacterium phoceense 60QC2CO	JANIKN000000000	29	2869146	63,31	303233	242887
Corynebacterium phoceense 70QC2O2	JANIKO000000000	52	2850545	63,28	94859	49232
Corynebacterium phoceense 92QC2O2	JANIKP000000000	42	2776783	63,38	247769	106288
Neisseria dentiae 1292RC1	JANIKQ000000000	65	2640494	54,46	111681	68038
Pediococcus pentosaceus 105QC2O2	JANIKR000000000	30	1897331	37,18	173352	79340
Pediococcus pentosaceus 111QC2O2	JANIKS000000000	28	1857120	37,15	217787	80396
Pelistega suis 138RC1	JANIKT000000000	20	2237786	41,83	367223	258956
Staphylococcus aureus 127RC1	JANIKU000000000	53	2817765	32,76	544928	109460
Staphylococcus aureus 131RC1	JANIKV000000000	50	2815128	32,76	545094	158584
Staphylococcus aureus 133RC1	JANIKW000000000	46	2814895	32,75	545554	110967
Staphylococcus aureus 137RC1	JANIKX000000000	50	2815652	32,75	545439	158584
Staphylococcus hyicus 40RC1	JANIKY000000000	27	2542706	35,49	218304	113518
Staphylococcus hyicus 147RC1	JANIKZ000000000	21	2530377	35,65	530306	329427
Staphylococcus hyicus 166RC1	JANILA000000000	20	2529675	35,64	530306	329139
Staphylococcus hyicus 188RC1	JANILB000000000	21	2530579	35,65	530306	329419

antiSMASH																
Betalactone	NRPS	NRPS-like	RiPP-like	Lanthipeptide-class-v	T3PKS	RaS-RiPP	lassopeptide	terpene	T1PKS	NAPAA	siderophore	cyclic-lactone-autoinducer	lanthipeptide-class-i	arylpolyene	resorcinol	hserlactone
	1							1								
	1							1						1		
	1							1								
								1	1	1						
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	1				1			1			2	1	1			
	1				1			1			2	1	1			
	1				1			1			2	1	1			
											2	1				
	1										2	1	1			
	1										2	1	1			
	1										2	1	1			

Supplementary Table 2. Continued.

		WGS info				
		# contigs	Total length (bp)	GC (%)	N50	N75
ID	Assembly Accession					
Staphylococcus hyicus 197RC1	JANILC000000000	21	2530891	35,65	530306	183933
Mammaliicoccus sciuri 8QC2O2	JANILD000000000	24	3113171	32,36	332168	302162
Staphylococcus gallinarum 32QC2CO	JANILE000000000	23	2862281	33,17	387557	221594
Staphylococcus cohnii 72QC2O2	JANILF000000000	22	2674187	32,59	1382822	270721
Staphylococcus borealis 63QC2O2	JANILG000000000	51	2482159	33,79	147891	59271
Streptococcus gallolyticus 151RC1	JANILH000000000	27	2263054	37,56	219868	80058
Streptococcus suis 12RC1	JANILI000000000	48	2157327	41,38	99724	56443
Streptococcus suis 15RC1	JANILJ000000000	43	2148663	41,17	112565	51845
Streptococcus suis 27RC1	JANILK000000000	98	2384308	41,4	60530	31497
Streptococcus suis 28RC1	JANILL000000000	101	2385480	41,41	61855	31497
Streptococcus suis 29RC1	JANILM000000000	103	2382005	41,41	60354	31497
Streptococcus suis 120RC1	JANILN000000000	62	2158422	41,46	99822	56249
Streptococcus suis 198RC1	JANILO000000000	96	2386023	41,41	60398	31497
Streptococcus suis 23QC2CO	JANILP000000000	44	2094952	41,05	106400	52611

antiSMASH																
Betalactone	NRPS	NRPS-like	RiPP-like	Lanthipeptide-class-v	T3PKS	RaS-RiPP	lassopeptide	terpene	T1PKS	NAPAA	siderophore	cyclic-lactone-autoinducer	lanthipeptide-class-i	arylpolyene	resorcinol	hserlactone
	1										2	1	1			
			1		1						1	1				
	1		1		1			2			1	1				
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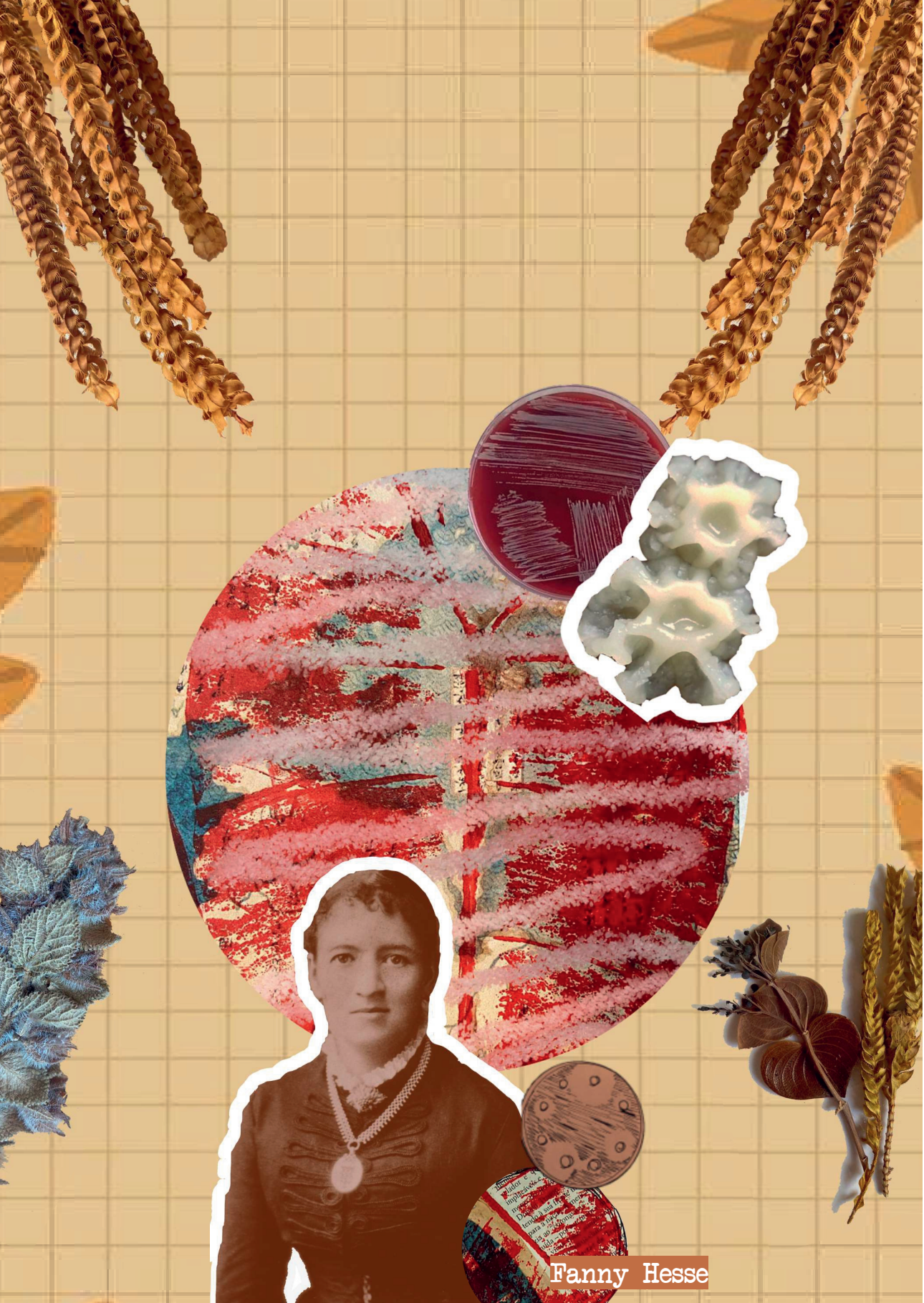
Fanny Hesse

IV

Comparative genomics of *Rothia* species reveals diversity in novel biosynthetic gene clusters and ecological adaptation to different eukaryotic hosts and host niches

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Abstract

Rothia species are understudied members of the phylum Actinobacteria and prevalent colonizers of the human and animal upper respiratory tract and oral cavity. The oral cavity, including the palatine tonsils, is colonized by a complex microbial community, which compete for resources, actively suppress competitors and influence host physiology. We analysed genomic data from 43 new porcine *Rothia* isolates, together with 112 publicly available draft genome sequences of *Rothia* isolates from humans, animals and the environment. In all *Rothia* genomes, we identified biosynthetic gene clusters predicted to produce antibiotic non-ribosomal peptides, iron scavenging siderophores and other secondary metabolites that modulate microbe– microbe and potentially microbe–host interactions. *In vitro* overlay inhibition assays corroborated the hypothesis that specific strains produce natural antibiotics. *Rothia* genomes encode a large number of carbohydrate-active enzymes (CAZy), with varying CAZy activities among the species found in different hosts, host niches and environments. These findings reveal com- petition mechanisms and metabolic specializations linked to ecological adaptation of *Rothia* species in different hosts.

Keywords: antimicrobials, carbohydrate-active enzymes, microbiome, *Rothia*, NRPS.

Impact statement

Rothia species are abundant members of the upper respiratory tract microbiota in humans and animals but are relatively understudied. Like other notable members of the Actinobacteria phylum, *Rothia* encode novel bioactive compounds in their biosynthetic gene clusters (BGCs) and are an untapped potential source of new antimicrobials. Additionally, our work advances knowledge about the genetics and physiology of *Rothia* species revealing correlations between BGCs, carbohydrate metabolic activities and the colonization of specific ecological niches.

Introduction

The oropharyngeal cavity including the palatine tonsils is colonised by a complex microbial community influencing pig health and physiology (1). *Rothia* bacteria (phylum Actinobacteria) are prevalent members of the upper respiratory tract microbiota of humans (2), pigs (1), rats (3) and birds (4) and early colonizers of the oropharyngeal cavity including the tonsils, tongue and teeth of healthy humans and piglets (5-7). It is therefore possible that specific *Rothia* species contribute to establishment of the microbial communities colonising healthy animals in early life. Although several different *Rothia* species have been described, there is little knowledge about genomics in relation to species physiology and their ecological niches.

Rothia bacteria are all Gram-positive rods and to date 14 species have been identified (https://www.bacterio.net/genus/rothia): *Rothia aeria* (8), *Rothia aerolata* (9), *Rothia amarae* (10), *Rothia arfidiae* (11), *Rothia dentocariosa* (12), *Rothia endophytica* (13), *Rothia halotolerans (14), Rothia koreensis (14), Rothia kristinae (14), Rothia marina* (15), *Rothia mucilaginosa (16), Rothia nasimurium* (16), *Rothia nasisuis* (17), and *Rothia terrae* (18). Furthermore, unclassified and uncultured *Rothia* genomes have been reconstructed from metagenomic data (19). Members of the *Rothia* genus, have been isolated from different ecosystems, including environmental samples as air and water, and host-associated niches including the skin, gut and oral cavity. Microbiota association studies have shown that the genus *Rothia* mainly correlates with healthy individuals (19-21) whereas specific strains from the species *R. aeria*, *R. dentocariosa* and *R. mucilaginosa* are associated with caries and dental pits, fissures and plaque (22, 23).

Recently, we showed that *Rothia nasisuis* colonizing the palatine tonsil epithelium of piglets produces the antimicrobial, antiviral and antiparasitic ionophore valinomycin *in vivo*, via a large multimodule non-ribosomal peptide synthetase (NRPS) enzyme complex encoded by genes tandemly arranged in a biosynthetic gene cluster (BGC) (24). Biosynthetic gene clusters (BGCs) produce diverse metabolites including natural antimicrobial products that fall into three major metabolite groups: post-translationally modified peptides (RiPPs), non-ribosomal peptides (NRPs) and polyketides (PKs) (25). RiPPs form a large heterogeneous group of peptides that are usually classified into post-translationally modified peptides and unmodified peptides (26). NRPs are relatively small peptides (2–30 amino acids) with hallmarks of nonproteinogenic amino acids and extensive" post-translational modifications". Substrates and intermediates are covalently bound during the assembly pathway and the order of the catalytic domains in the NRPS often parallels the order of their biosynthetic pathway (27, 28). Finally, PKs are categorized into different classes based on their biochemical mechanisms and enzyme architecture (29).

The presence of specific BGCs in *Rothia* species can be used as markers to group and distinguish between species, and BGC annotations help to predict ecological traits.

Carbohydrate-active enzymes (CAZy) are widely present in all organisms and play important roles in biological processes and ecological adaptations. CAZy are classified into several families and their annotation can be used to predict the ability of organisms to assemble and break down complex carbohydrates (30), important ecological traits for niche exploitation and colonization of novel environments. Bacteria colonizing different environments, display a range of CAZy metabolic capacities linked to the diversity, structure, and composition of carbohydrates available in the habitat (31). *Rothia* species in the oral microbiota have been reported as nitrate-reducers (32, 33). The conversion of nitrate to nitrite and nitric oxide can promote oral health by reducing the acidification of the saliva and inhibiting species contributing to periodontal disease through the antimicrobial activities of nitric oxide. Furthermore, nitrite is swallowed and taken up into the blood circulation where it is converted into nitric oxide. a signaling molecule which is reported to improve cardiovascular and metabolic health (32, 34).

Here, we report on genomic analyses of 43 novel *Rothia* strains that we isolated and cultured from the tonsillar microbiota of piglets between one and three weeks of age. To place the genomic and phylogenetic analysis of these novel porcine strains in a broader ecological context, we analysed these *Rothia* strains together with publicly available draft genome sequences from human, animal and environmental *Rothia* strains. We inferred phylogenetic trees based on genomic data that we annotated with origin (niche) and presence/absence of genes of interest including biosynthetic gene clusters, carbohydrate-active enzymes, and antibiotic resistance and nitrate-nitrite metabolism genes, in order to discover ecological adaptations of *Rothia* species to different mammalian hosts (pigs and human) and other environments.

Material and Methods

1. Samples

We sampled the palatine tonsil of six random piglets at two high-health status farms in Catalunya, Spain, one week before weaning (timepoint -1) and three weeks after weaning (timepoint +3). Tonsil biofilms were collected using Puritan HydraFlock Swabs and stored at -80°C in transport medium (buffered peptone +15% (v/v) glycerol).

2. *Rothia* spp isolation, identification and culture conditions

Dilutions of the tonsil samples between 10^{-1} and 10^{-3} were plated for each sample onto Sheep Blood Agar (35) (Becton Dickinson, Heidelberg, Germany) and Brain Heart Infusion Agar (36) (Becton Dickinson) media. Single isolates were selected based on the morphology (size, shape and colour) of the colonies and transferred to BHI liquid medium (Supplementary Figure 1), incubated at 37°C in presence of 5% CO2. Isolates were purified using Gram staining and the quadrant streak plate method (37) and then stored at -80°C into cryotubes containing BHI and 15% (v/v) glycerol.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was used for screening and identification of pure colonies. The samples were spotted in the MALDI-TOF MS target plate and covered with 1 µL of matrix solution (saturated α-cyano acid-4-hydroxycinnamic in 50% acetonitrile and 2.5% trifluoroacetic acid) (38). The samples were analysed using a MICROFLEX spectrometer (Bruker Daltonics) according to the manufacturer's recommendations. For each spectrum, 90-100 peaks were compared with reference databases at the Department of Medical Microbiology, University Medical Centre Groningen, the Netherlands. An isolate was considered correctly identified at the species level when at least one MALDI-TOF MS spectrum score was ≥1.9 and at the genus level with a score ≥1.7 (38).

3. Bacterial indicator strains and culture conditions

The following culture media and bacteria were used in overlay inhibition assays (Table 1) to identify *Rothia* isolates with antagonistic activity.

4. Overlay inhibition assays

Rothia isolates were cultured in Brain Heart Infusion (BHI) broth medium and incubated overnight at 37°C. Using a 96-well replicator (Boekel Scientific), overnight cultures of *Rothia* were spotted onto BHI agar media in Petri dishes (Supplementary figure 1) and grown for 18 hours. Bacterial colonies were inactivated by exposure to UV light for 20 min and overlayed with 20 mL soft agar (0.75% w/v agar) containing approximately $1x10^5$ colony forming units (CFU)/mL of the indicator bacteria (Table 2). Antimicrobial activity was determined by the presence of visible zones of growth inhibition ("halos") around the *Rothia* colonies after overnight incubation (39).

5. Genomic DNA extraction and sequencing

Rothia species and strains were grown overnight in BHI broth at 37°C. The next day, cells were pelleted by centrifugation, and genomic DNA was extracted using the PowerSoil Genomic Purification Kit (Qiagen) according to the manufacturer's

Table 1. Indicator bacteria and culture conditions

Indicator bacteria	Source/strain	Culture medium/ temp (°C)
Streptococcus suis	P1/7	THB/37 °C
Streptococcus suis	S10	THB/37 °C
Streptococcus suis	J28	THB/37 °C
Staphylococcus aureus	ATCC 6538P	BHI/37 °C
Escherichia coli	L 4242	LB/37 °C
Streptococcus porci	DSM 23759	THY/37 °C
Streptococcus parasuis	DSM 29126	THY/37 °C
Streptococcus porcinus	DSM 20725	THY/37 °C

DSM, Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures; ATCC, American Type Culture Collection; L prefix are from the NAICONS pathogens library, Italy. The strains of *S. suis* strain P1/7 (36), *S. suis* strain S10 (37) are zoonotic pathogens and the *S. suis* strain J28 (38) is less pathogenic, an unencapsulated mutant. *Escherichia coli* L4242, a *ΔtolC* mutant derivative from MG1061 (39). THB, Todd Hewitt broth (Becton Dickinson); BHI, Brain Heart Infusion (Becton Dickinson); LB, Luria-Bertani (Becton Dickinson); THY; THB enriched with 0.2% (w/w) yeast extract.

recommended protocol for Gram-positive bacteria. Recovery of high molecular weight DNA was assessed on a 0.8% agarose gel (Sigma-Aldrich) in 1X TAE buffer [Tris-HCl 40 mM, 20 mM acetic acid and 1 mM EDTA (pH 8)], stained with 25 µg/mL of SYBR Safe and quantified using the Qubit dsDNA Broad-Range (BR) assay and Invitrogen's Qubit Fluorometer.

6. Genome sequencing, assembly, annotation and phylogenetic analysis

We obtained genome sequences from 43 tonsil isolates of *Rothia* from five piglets, randomly selected from different litters on two farms with a high-health status. Genome sequencing was performed on an Illumina HiSeq 2000 platform (Illumina) at MicrobesNG, Birmingham, United Kingdom. However, due to lower sequencing depth and shorter read lengths, a few genome sequence assemblies had a high number of contigs (Supplementary Table 3). Reads were trimmed using Trimmomatic 0.30 software with a sliding window set at Q15 (40). Genome assembly was performed using SKESA 2.4.0 (41). In total, 112 genome sequences of publicly available *Rothia* species were downloaded from NCBI's Reference Sequence database (Supplementary table 1) (download 27/04/2021). All genomes were annotated using Prokka 1.14.6 (42). The "core" genes were determined based on 90% protein similarity using Roary 3.13.0 (43) (Supplementary table 2). A maximum-likelihood tree was inferred using IQ-TREE 2.1.4 on default parameters from the core gene alignment obtained from Roary 3.13.0. Tree visualisation was done in R 4.0.5 using ggtree 2.4.2 (44).

7. Carbohydrate-active enzyme analysis.

Carbohydrate-active enzymes (CAZy) present in the genomes were determined using dbCAN2 2.0.11 using default parameters (45). CAZy were annotated as auxiliary activities (AA), carbohydrate-binding modules (CBM), carbohydrate esterase (CE), glycoside hydrolases (GH), polysaccharide lyases (PL), or glycosyl-transferases (GT). To limit false positives, a minimum of two hits were considered reliable. Hierarchical clustering based on Bray-Curtis dissimilarity was used to cluster the genomes. Five clusters were determined to be the optimal number based on gap statistics. Principal component analysis (PCA) was used to visualise the distribution of the CAZy across the collection genomes in our dataset.

8. Antimicrobial resistance genes (ARGs) and Nitrate-Nitrite metabolism genes

Antimicrobial resistance genes (ARGs) were identified using Abricate 1.0.1 (https://github.com/tseemann/abricate) with the ResFinder database (36) at 80% DNA identity and coverage cut-off for both. The sequences taxonomically related to nitrate/nitrite metabolism in Rothia (32) were compared by similarity using the program BLASTx (45) against all the genomes used in this study to identify the potential hits with filtering of e-value <10−5 and as threshold for sequence identity and coverage ≥50%.

9. Mining of antimicrobial peptide encoding genes

For identification and annotation of biosynthetic gene clusters (BGCs), draft genomes of all *Rothia* strains were analysed using antiSMASH 6.0.1 using default detection criteria for the analysis (47). The BGCs from the strains were sorted into groups based on their predicted activity. In addition, the Biosynthetic Genes Similarity Clustering and Prospecting Engine (BiG-SCAPE) software was used to define a distance metric between gene clusters using a combination of three indices: Jaccard Index of domain types, Domain Sequence Similarity, and the Adjacency Index (48). Networks were visualised using Cytoscape 3.8.2 (49). Clinker 0.0.21 was used for the alignment and comparison of BGCs (50).

Results

Rothia spp. isolation and identification corroborated and complemented previous Rothia distribution reports

To obtain upper respiratory isolates of the Rothia genus from pigs we cultured tonsil swabs from five piglets on sheep blood agar plates and purified over 500 isolates by repeated steaking of single colonies on fresh plates. Ninety-three isolates were identified as *Rothia* spp by MALDI-TOF MS analysis (data not shown). Forty-six *Rothia* isolates were then selected for genome sequencing based on differences in colony morphology, growth characteristics and inhibitory activity against different target bacteria in the same niche. The assembled genomes ranged between 2.31–2.79 Mb in size, with a GC-content between 56.7-60.3% (Supplementary table 3). We identified 43 *Rothia* isolates to the species level using full-length sequences of 16S small subunit ribosomal RNA (rRNA); *R. nasimurium* (n=36), *R. nasisuis* (n=5), *R. aerolata* (n=1) and *R. endophytica* (n=1).

Genomic analyses and screening the genomic dataset to discover candidate genes of interest in colonization and adaptation of the different species in the environment

To identify accessory genes involved in antibiotic resistance, secondary metabolite biosynthesis and carbohydrates utilization genes which might play crucial roles in niche adaptation, competition, and persistence, we analysed the genomes of our 43 isolates and 112 publicly available draft genomes of *Rothia* species and strains (as of April 2021). In total, 155 *Rothia* genomes were included along with the metadata, sample origin and disease-associated or commensal species. (Figure 1). The relatedness of the 155 genomes was investigated using core-genome phylogenies and mined for the presence of (i) biosynthetic gene clusters (BCGs), (ii) antimicrobial resistance genes, and (iii) carbohydrate-active enzymes (CAZy) encoded in the genome.

We constructed phylogenetic trees based on 90% core protein similarities (encoded by 28 core genes that were shared by the *Rothia* strains (Supplementary Table 2) and observed three major clades that overall corresponded to their origin; human, environmental/food and, porcine/other animals (sponge, *Mus musculus*, duck and marmot). We annotated the phylogeny with the presence/absence of different classes of BGCs and antibiotic resistance genes (Figure 2).

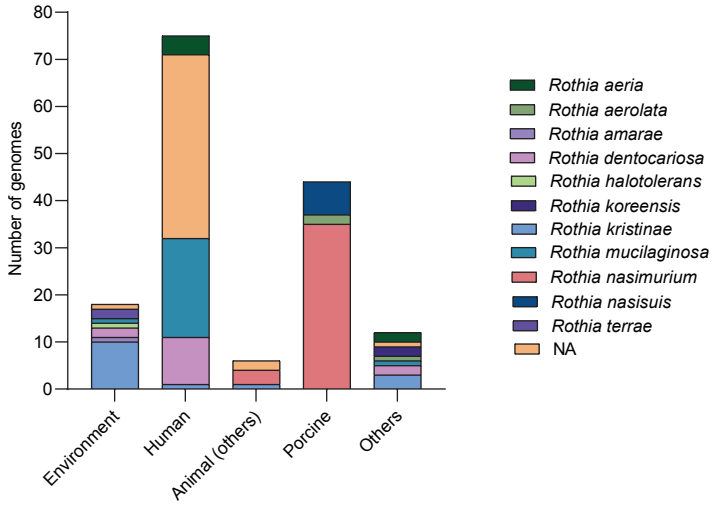


Figure 1. *Rothia* genomes including the species *R. aerolata, R. aeria, R. amarae, R. kristinae, R. dentocariosa, R. halotolerans, R. mucilaginosa, R. terrae, R. nasimurium, R. nasisuis, R. koreensis* and *Rothia* sp identified in metagenomic data from mainly human clinical samples in the NCBI Reference Sequence Database. The bar chart shows the number of genomes analysed for each Rothia species per sampling site (i.e. origin of isolates); 'others' correspond to isolates from food and unspecified samples. Animal (others) corresponds to diverse animal groups (sponge, *Mus musculus*, duck and marmot) but not pigs. NA no assigned species (*Rothia* sp).

Genome mining for antibiotic resistance, nitrate/nitrite reduction genes and BGCs

On average *Rothia* genomes contain between 2 to 8 antibiotic resistance genes (ARGs) including variants of the gene "families" *aac, aad, ant, aph, cmx, dfr, erm, sul* and *tet*. ARGs were identified mainly in *Rothia* isolates belonging to the species *R. nasimurium* and *R. mucilaginosa* sampled from pigs and humans (Figure 2). Most of the *Rothia* genomes encode genes involving nitrate/nitrite metabolism as previously reported (51). The predicted gene functions and number of genes associated with nitrate/nitrite reduction vary across *Rothia* species and their ecological niche (Supplementary Table 5). Human-associated *Rothia* species carry the largest number of genes involved in nitrate and nitrite reduction metabolism, followed by animal-associated *Rothia* species.

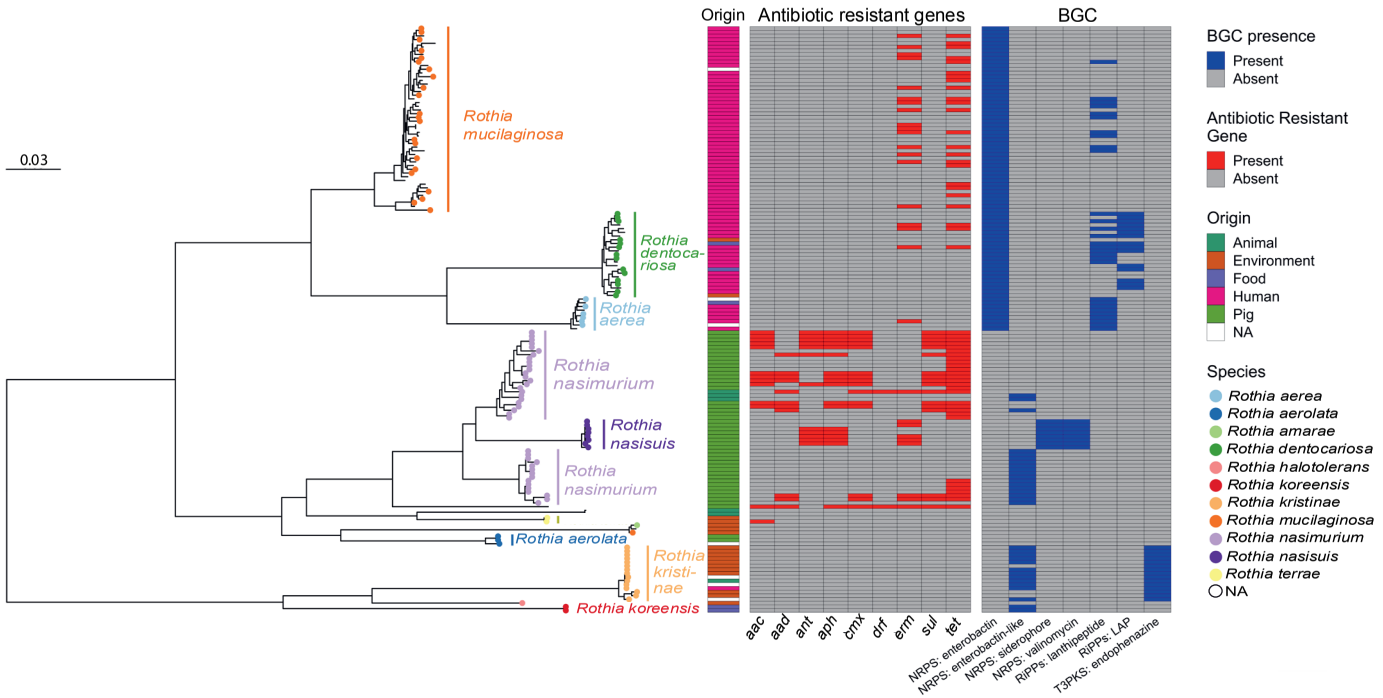


Figure 2. Overview of the biosynthetic gene clusters (BGCs) and antimicrobial resistance genes (ARG) in the species of *Rothia* analysed in this study from different sampling sites. ARG represents multiple variants of the gene 'families'. The compilation follows: the sampling-sites are represented by the colours shown in the inner colourful column. The rooted tree was constructed based on the core genes (n=28), the species are named and highlighted with the same colour. The antibiotic resistance genes are displayed as presence (red) and absence (grey), which could lead to the selection of potential producers of bioactive compounds. The outermost columns display the presence/absence of biosynthetic gene clusters; distinct clusters were annotated according to antiSMASH in grey (absence) and dark blue (presence). The scale bar indicates nucleotide substitution per site.

Overall, 386 BGCs were predicted in the 155 genomes used in this study. In 80% of the *Rothia* genomes, at least one BGC was annotated as non-ribosomal peptide synthetase (NRPS) or hybrid non-ribosomal peptide-polyketide (NRP/PK) synthase, which together account for 146 of all the predicted clusters (Figure 2). Other common BGC types were gene clusters annotated as producing betalactone; multidomain polyketide synthases (PKSs), and ribosomally synthesized, post-translationally modified peptides (RiPPs) with a broad range of biological functions including antimicrobial activity (Supplementary table 3).

In the 43 porcine Rothia isolates, antiSMASH mainly predicted the presence of NRPs, RiPPs and NRPs (Table 2). NRPS gene cluster (family FN2043), found only in *R. nasisuis* is predicted to produce the natural antibiotic valinomycin (Figure 3C). Genomes of *R. nasimurium,* possess NRPs clusters (family FN0062); members of this family are predicted to produce secondary metabolites (Figure 3A). These 18 BGCs had low similarity showing potential novelty based on the BigScape analyses.

Overlay inhibition assays

Inhibition assays were performed using a panel of eight indicator bacteria with all *Rothia* strains from this study (n=43) (Table 2). The indicator bacteria included strains of three zoonotic pathogen *S. suis,* which colonises the oropharyngeal cavity and tonsil epithelium of pigs, as well as related streptococci *S. porci, S. porcinus, S. parasuis, S. aureus* and *E. coli* as a representative Gram-negative bacterium (Table 1). Fourteen *Rothia* strains were able to inhibit the growth of at least one species of Gram-positive bacteria tested (Table 2). All *R. nasisuis* and six strains of *R. nasimurium* showed growth inhibitory activity against streptococci but had no activity against *S. aureus* and two strains of *R. nasimurium* had low activity against the Gram-negative *E. coli.*

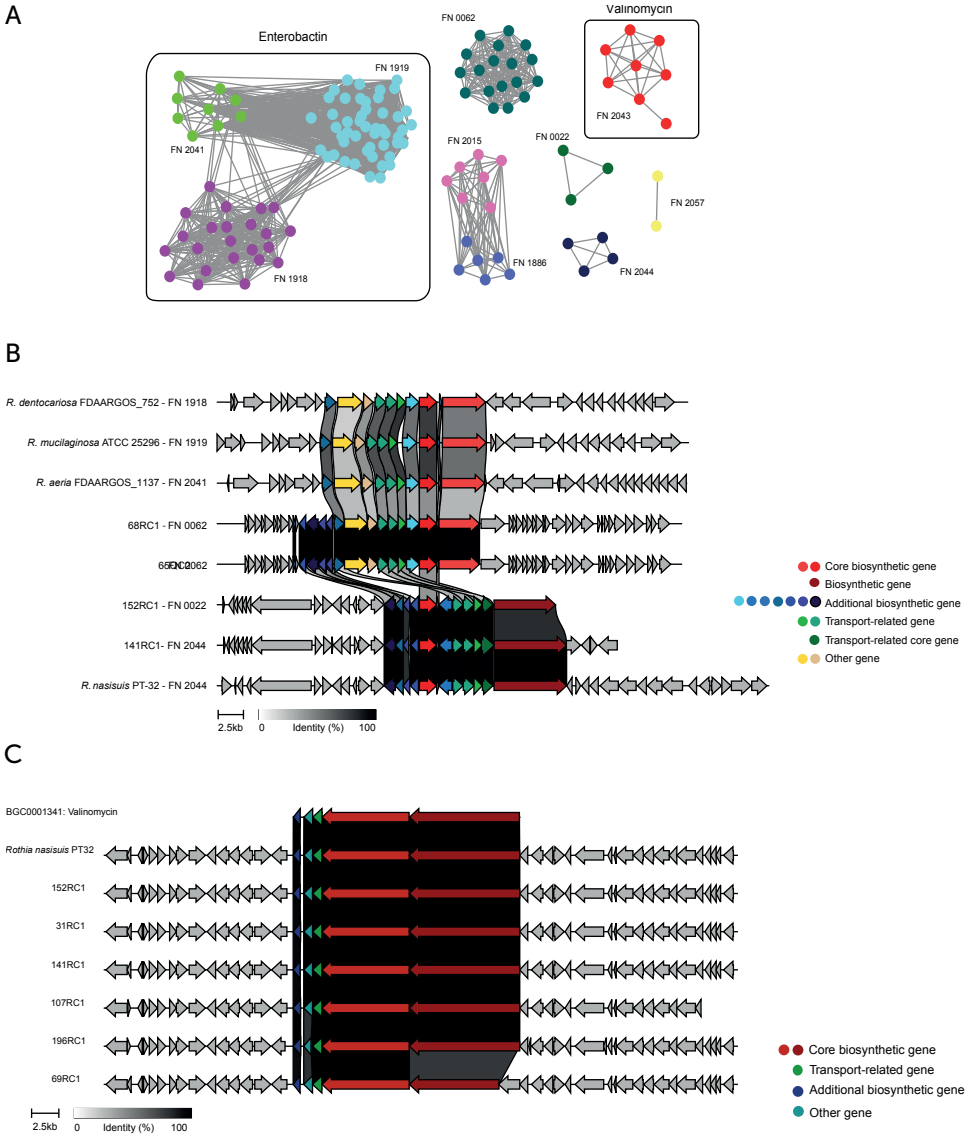


Figure 3. Overview of NRPs-type BGCs from *Rothia* species. **(A)** Similarity network of known and putative BGCs annotated as NRPs based on antiSMASH results. The small groups of NPRS families (FN) with unknown clusters containing fewer common genes are drawn outside the boxes: FN2015, FN0022, FN2044 and FN1086. Highlighted groups include widely distributed clusters in many different species. The network containing red nodes represents the valinomycin (FN2043) cluster that was present in all porcine *R. nasisuis* isolates. The network containing

light blue, green and purple nodes represent BGCs corresponding to Enterobactin cluster families FN1918, FN1919, and FN2041 that were present in the species of *R. mucilaginosa, R dentocariosa* and *R. aeria* that were mainly sampled from the upper respiratory tract of humans. The network containing dark green nodes represents an NRP-like cluster annotated as family FN0062 that was prevalent in *R. nasimurium*; this cluster contained conserved genes from the Enterobactin cluster. The families represent the group of BGCs that encode biosynthesis of highly similar or identical metabolites classified in arbitrary numbers based on the outcome of the analyses. **(B)** Overview of representative species that contain conserved Enterobactin BGCs, or NRP-like cluster annotated as family FN0062 (*R. nasimurium*) that shares genes with Enterobactin BGC as well as the families FN0022 and FN2044. **(C)** Overview of the conserved valinomycin cluster (BIG-SCAPE family FN2043) in the species *R. nasisuis*. BIGSCAPE output is visualized as a network using Cytoscape and the cluster organization visualized using Clinker (https://github.com/gamcil/clinker).

Carbohydrate Active enZymes (CAZy)

We discovered more than 5,040 diverse Carbohydrate Active enZyme (CAZy) genes which were classified in different families and subfamilies (Supplementary table 4), suggesting there may be a link between CAZy functionality ("toolbox") and the ability to colonise and persist in novel niches under varying environmental conditions. Using the gene annotations of *Rothia* species*,* our analyses revealed that *Rothia* species contain five major families of genes predicted to participate in carbohydrate metabolism and energy conversion systems, including genes predicted to encode glycosyl hydrolases (GHs), glycosyl transferases (GT), carbohydrate-binding modules (CBM), carbohydrate esterases (CE), auxiliary activities (36), and other carbohydrate metabolic pathway components (Figure 4).

Overall, 41 CAZy families were annotated among the *Rothia* genomes (21 GHs, 14 GTs, 2 CBMs, 3 CE and one AA) at different abundances per species and niche (Figure 4). The GHs had highest gene sequence diversity and abundance followed by the GTs. Rothia species have large numbers of GTs representing >65% of the total CAZy enzymes toolbox. GT families are highly diverse in the CAZy database but in the *Rothia* genomes just two families, GT2 and GT4, represent a large portion of the total number of GT genes (n=3372) identified all strains and niches. The first horizontal blocks of the heatmap (Figure 4) shows clustering between the families CBM5-GT87*,* which contains enzymes prevalent in several species of *Rothia* in all niches, suggesting the presence of essential enzymes responsible for carbohydrate metabolism. The CAZy families GH33, GH36, GT5, GT35, CBM48+GH13-11 and CBM48+GH13-9 (CAZy Group 1) were mostly discovered in the animal-associated species *R. nasimurium* and *R. nasisuis*. The families GT25 and CBM50+GH23 were more often identified in human-associated *Rothia* species; these enzymes clustered

Table 2. *In vitro* inhibitory activity of the 43 *Rothia* sp. obtained from porcine palatine tonsil.

		Indicator bacteria								
ID	Species	Streptococcus suis *J28*	Streptococcus suis *S10*	Streptococcus suis *P1/7*	Streptococcus porci *DSM 23759*	Streptococcus porcinus *DSM 20725*	Streptococcus parasuis *DSM 29126*	Sthaphylococcus aureus *L100*	Escherichia coli *L4242*	BGCs from WGS (AntiSMASH 6.0)
159RC1	*Rothia aerolata*	-	-	-	-	-	-	-	-	Betalactone/RRE-containing
56QC2O2	*Rothia endophytica*	-	-	-	-	-	-	-	-	*Betalactone/RRE-containing/RiPPs /terpene*
63RC1	*Rothia nasimurium*	-	-	-	-	-	-	-	-	Betalactone/RRE-containing/
67.3RC1	*Rothia nasimurium*	-	-	-	-	-	-	-	-	Betalactone/NRPS
68RC1	*Rothia nasimurium*	-	-	-	-	-	-	-	-	Betalactone/NRPS
110RC1	*Rothia nasimurium*	+/-	-	-	+/-	-	-	-	-	NRPS
136RC1	*Rothia nasimurium*	-	-	-	+/-	-	-	-	-	NRPS
207RC1	*Rothia nasimurium*	-	-	-	-	-	-	-	-	NRPS
8QC2CO	*Rothia nasimurium*	-	-	-	-	-	-	-	-	Betalactone/RRE-containing
15QC2CO	*Rothia nasimurium*	-	-	-	-	-	-	-	-	Betalactone/RRE-containing
22QC2CO	*Rothia nasimurium*	-	-	-	-	-	-	-	-	Betalactone/RRE-containing
28QC2CO	*Rothia nasimurium*	-	-	-	-	-	-	-	-	Betalactone/RRE-containing
38QC2CO	*Rothia nasimurium*	-	-	-	-	-	-	-	-	Betalactone /RRE-containing
48QC2CO	*Rothia nasimurium*	-	-	-	-	-	-	-	-	Betalactone/RRE-containing
54QC2CO	*Rothia nasimurium*	-	-	-	-	-	-	-	-	Betalactone/RRE-containing /RiPP-like
64QC2CO	*Rothia nasimurium*	-	-	-	-	-	-	-	-	Betalactone/RRE-containing /NRPS

2QC2O2	*Rothia nasimurium*	-	-	-	-	-	-	-	-	Betalactone/RRE-containing
3QC2O2	*Rothia nasimurium*	-	-	-	-	-	-	-	-	Betalactone/NRPS
4QC2O2	*Rothia nasimurium*	-	-	-	-	-	-	-	-	Betalactone/RRE-containing /RiPP-like
6QC2O2	*Rothia nasimurium*	-	-	-	-	-	-	-	-	Betalactone/RRE-containing /RiPP-like
7QC2O2	*Rothia nasimurium*	-	-	-	-	-	-	-	-	Betalactone/RRE-containing
19QC2O2	*Rothia nasimurium*	-	-	-	-	-	-	-	-	Betalactone/RRE-containing
34QC2O2	*Rothia nasimurium*	-	-	-	-	-	-	-	-	Betalactone /NRPS
36QC2O2	*Rothia nasimurium*	-	-	-	-	-	-	-	-	Betalactone/RRE-containing/RiPP-like
37QC2O2	*Rothia nasimurium*	-	-	-	-	-	-	-	-	Betalactone/RRE-containing/RiPP-like
46QC2O2	*Rothia nasimurium*	-	-	-	-	-	-	-	-	Betalactone/RRE-containing/RiPP-like
63QC2CO	*Rothia nasimurium*	-	-	-	-	-	-	-	-	Betalactone/RRE-containing
65RC1	*Rothia nasimurium*	-	-	-	-	-	-	-	-	Betalactone/NRPS
65.2RC1	*Rothia nasimurium*	-	-	-	-	-	-	-	-	Betalactone/NRPS
66RC1	*Rothia nasimurium*	-	-	-	-	-	-	-	-	NRPS
67RC1	*Rothia nasimurium*	-	-	-	-	-	-	-	-	Betalactone/NRPS
123RC1	*Rothia nasimurium*	+/-	-	-	+/-	-	-	-	-	NRPS
124RC1	*Rothia nasimurium*	+/-	-	-	+/-	-	-	-	-	NRPS
213RC1	*Rothia nasimurium*	-	-	-	-	-	-	-	-	Betalactone/RRE-containing
206RC1	*Rothia nasimurium*	-	-	-	-	-	-	-	-	NRPS
15QC4O2	*Rothia nasimurium*	-	-	-	-	-	-	-	+	RRE-containing
18QC4O2	*Rothia nasimurium*	-	-	-	-	-	-	-	+	RiPP-like/RRE-containing
31RC1	*Rothia nasisuis*	+	+/-	+	+	+	+	-	-	Betalactone/RRE-containing/NRPS (valinomicin)
69RC1	*Rothia nasisuis*	-	-	+	+	+	+	-	-	Betalactone/RRE-containing/NRPS (valinomicin)
141RC1	*Rothia nasisuis*	+	+/-	+	+	+	+	-	-	Betalactone/RRE-containing/NRPS (valinomicin)
152RC1	*Rothia nasisuis*	+	+/-	+	+	+	+	-	-	Betalactone/RRE-containing/NRPS (valinomicin)
196RC1	*Rothia nasisuis*	+	+/-	+	+	+	+	-	-	Betalactone/RRE-containing/NRPS (valinomicin)
107RC1	*Rothia nasisuis*	+/-	-	-	-	-	-	-	-	Betalactone/RRE-containing/NRPS (valinomicin)

Inhibition zones were scored after overnight incubation in at least two assays. (–) absence of inhibitory activity; (+/-) inhibition zone between 3 and 6 mm diameter (weak); (+) inhibition zone ≥7 mm diameter (14). NRPS: non-ribosomal peptide synthetase; RRE-containing: recognition element containing NRPs and/or PKs clusters; RiPPs-like: category of ribosomally synthesized and post-translationally modified peptides.

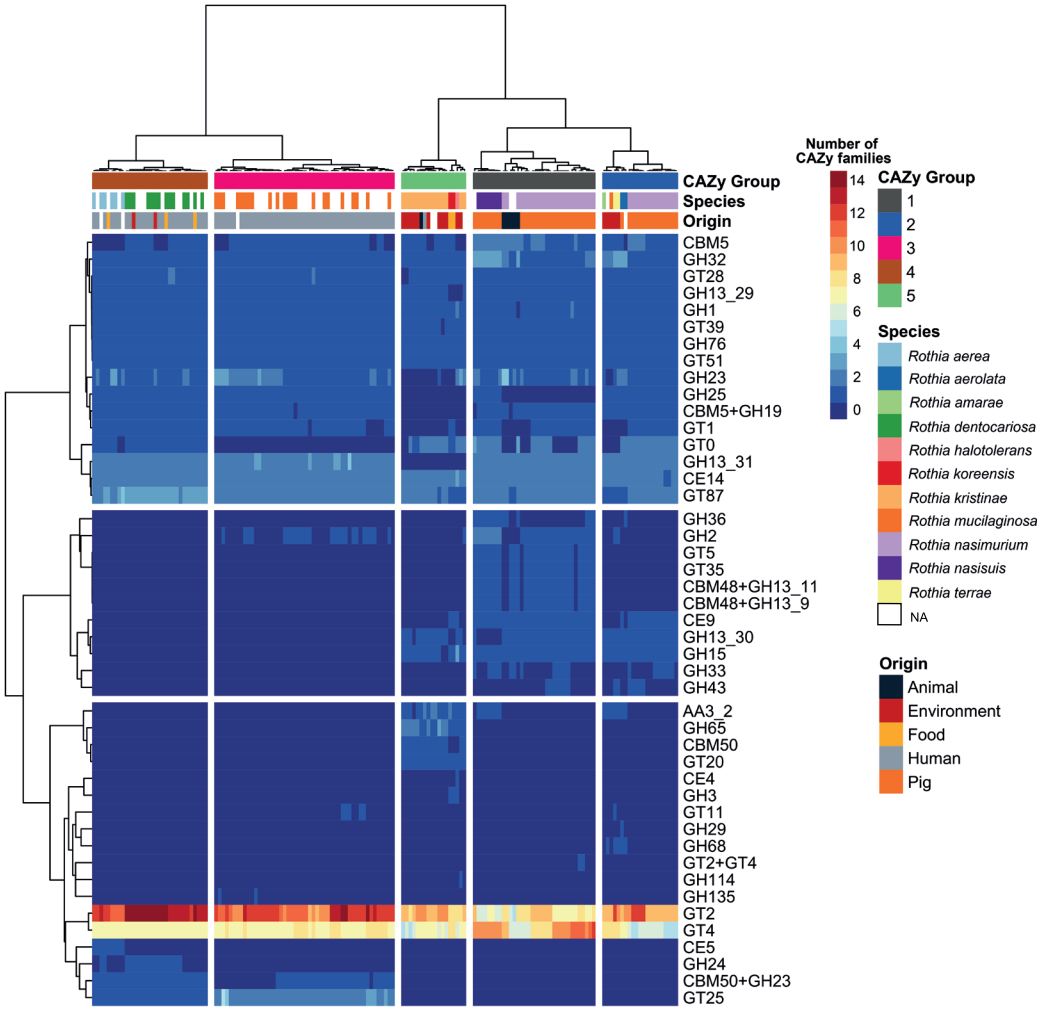
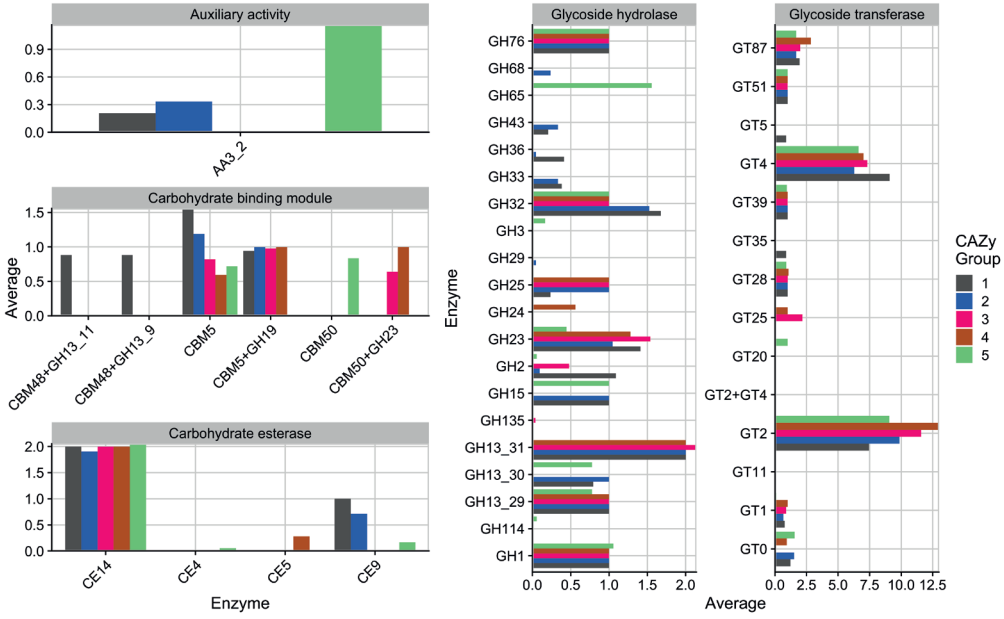


Figure 4. Heatmap displaying presence/absence of CAZy families across *Rothia* genomes. The heatmap includes data from 41 CAZy families (rows); the sampling site and distribution of CAZy families across *Rothia* species are displayed in bars above the heatmap. 'CAZy group' (columns) represents the enzymes present in a specific group of species clustered according to our analysis. All displayed CAZy gene data and annotations were retrieved from the CAZy enzyme database (accessed October 2021). Glycosyl hydrolase (GH), glycosyl transferases (GT), carbohydrate-binding modules (CBM), carbohydrate esterases (CE).

in CAZy groups 2 and 3. Specifically, the family GT25 was mainly discovered in the genomes of *R. dentocariosa* and the family complex CBM50+GH23 was present in 85% of the *Rothia* species isolated from human microbiota. On the other hand, CAZY enzymes GH65, CBM50, and GT20 were only discovered in *Rothia* species sampled from the environment (Figure 5).



CAZy group	Species	Sampling site
1	*R. nasisuis*, *R. nasimurium* and NA	Porcine and other animals
2	*R. nasimurium*, *R. aerolata*, *R. amarae*, *R. terrae*, *R. mucilaginosa* and NA	Environment, porcine and NA
3	*R. mucilaginosa* and NA	Human and NA
4	*R. aerea*, *R. dendocariosa* and NA	Human, Environment, food and NA
5	*R. kristinae*, *R. koreensis* and *R. halotolerans*	Environment, other animals, human, food and NA

Figure 5. Overview of CAZy enzyme families prevalent in the Rothia genus. Based on the distribution of species and sampling origins, CAZy enzymes were clustered in five groups displayed in the first column of the table displayed at the bottom. Glycosyl hydrolases (GHs), glycosyl transferases (GT), carbohydrate-binding modules (CBM), carbohydrate esterases (CE). The bottom table is the overall of the 'CAZy group' that represents the enzymes present in a specific group of species clustered according to our analysis (Figure 4).

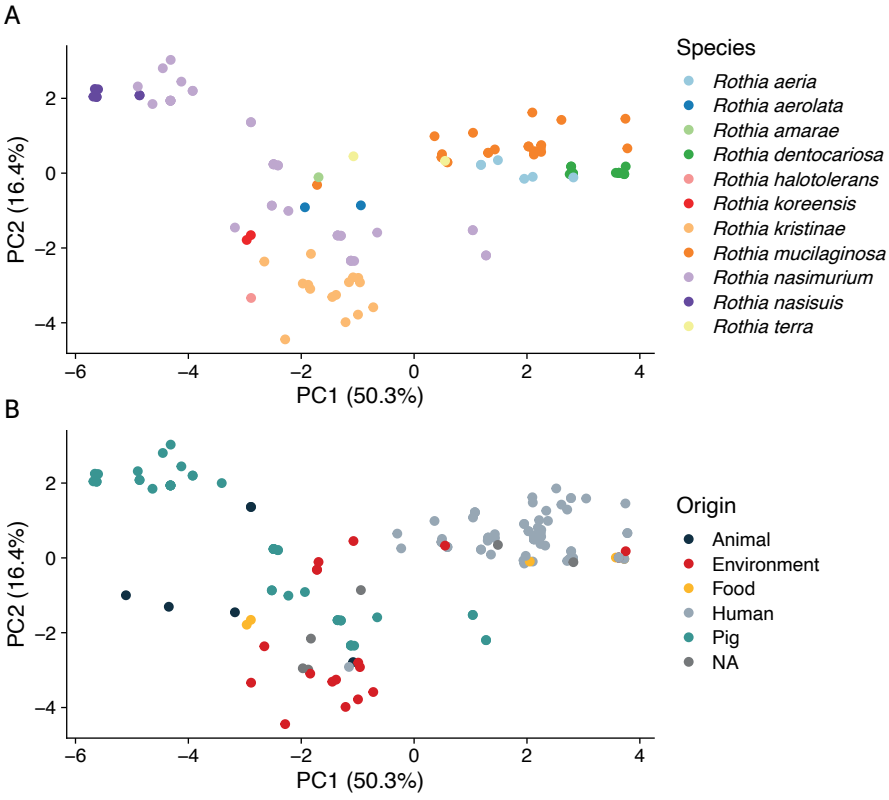


Figure 6. Principal component analysis (PCA) score plot of carbohydrate active enZyme (CAZy) distribution. The first two axes explain 50.3 and 16.4% of the total variance, respectively. The ecological traits are indicated by **(A)** taxonomic groups and **(B)** sampling sites.

To investigate CAZy distribution across the 155 *Rothia* genomes representing 12 species originating from different niches, a principal component analysis (PCA) was conducted (Figure 6). The first two principal components explained 66.7% (50.3% and 16.4% for PC1 and PC2, respectively) of the total variance.

The CAZy-based PCA plot clustered species largely by taxonomy; isolates and genomes from the species (Figure 6A). *R. mucilaginosa* and *Rothia* sp. (unclassified *Rothia*'s) mainly clustered in the upper right area. Most isolates and genomes of the species *R. kristinae, R. halotolerans* and *R. koreensis* clustered in the lower-left area. Most isolates and genomes of the species *R. aerea* and *R. aerolata* clustered in the middle. Finally, most isolates and genomes of the species *R. nasimurium* and *R.*

nasisuis clustered in the upper left area of the plot. Those *Rothia* species clustering together in the PCA also clustered closely in the phylogenetic analysis or colonized very similar niches (Figure 6B).

Discussion

Recently, *Rothia* was listed as no. 17 in the top 20 most abundant and prevalent bacterial genera discovered in the human oral cavity (2). Our recent work on porcine tonsil microbiota revealed *Rothia* as one of the most prevalent genera with at least 0.98% mean abundance across individuals; the genus was detected in more than 98% of the piglets from a data set with over 160 samples, across 11 farms sampled in Europe (unpublished).

The habitat specificity of *Rothia* species suggest adaptation to certain oral niches such as the teeth surface and the oral mucosa (2). Here we characterised the genomes of several species of the poorly characterised genus *Rothia* to gain a better understanding of the interactions and colonization in the upper respiratory tract and the potential ecological role of specialized metabolites and CAZy enzymes. Genome mining of the sequences from the databases of over hundred *Rothia* genomes and the recent sequenced porcine isolates identified several BGCs with potential to produce natural antimicrobial compounds. The metabolites predicted to be produced by these gene clusters include compounds that function in competitive interactions between microbes such as antibiotics and siderophores, but also compounds that may function in the host, including immunosuppressants and anticancer drugs. Potentially, the NRPS, PKS, and hybrid NRPS-PKS gene clusters could produce groups of bioactive compounds that form a source of antimicrobial products with broad applications (52). NRPs, PKs as well as other compounds produced by BGCs are released into the surrounding environment upon their biosynthesis. The synthesis is regulated by proteins that are activated by physical and environmental factors, including carbon and nitrogen source availability (52). Once released, the molecules produced by NRPSs and PKSs or their hybrid gene clusters play roles in niche colonisation, including optimisation of bacterial proliferation, *quorum sensing* activity, and providing higher substrate affinity or faster growth rates (53).

Valinomycin, identified only in *Rothia nasisuis* species, is an NRP ionophore, forming ion channels in membranes that allow free movement K^+ ions thereby, altering membrane potential that may lead to disruption of the normal K^+ ion membrane gradient (24, 54). All isolates of *R. nasisuis* containing the valinomycin-producing

NRPs showed inhibitory activity *in vitro* against Gram-positive target bacteria including different species of *Streptococcus* sp. and closely related *Rothia* species as previous reported (55) (Table 2). The presence of the valinomycin clusters in *R. nasisuis* likely provide this species with a competitive advantage to prevail in the porcine tonsil microbiota and influence the composition of the microbiota in the first weeks of piglet life (7, 54). We identified 18 novel NRPS gene clusters in the genomes of *R. nasimurium* species using BigScape. These clustered as family FN0062 and contain genes also present in the FN1919, FN1918 and FN2041 families that have been predicted to produce enterobactins, with strong binding affinity for ferric iron (56) (Figure 3B). Iron availability is scarce at mucosal surfaces due to host iron-sequestering proteins (57). NRPS-like clusters from family FN0062 are therefore likely involved in the acquisition of iron from the environment and facilitate colonisation of the human oral cavity by *R. mucilaginosa* and *R. dentocariosa* (58). Six other NRPSs were identified that might produce antimicrobials as judged by growth inhibition of Gram-positive and Gram-negative bacteria by *R. nasimurium in vitro*. However further research would be needed to verify this conclusion. We also report the first endophytic *Rothia* isolate originating from pig tonsil microbiota. *R. endophytica* has been found within healthy plant tissues (13) thus it is possible that the *R. endophytica* strain we isolated from the piglet tonsil swab originated from ingested plant material. Genomes of *Rothia* species (i.e. *R. nasimurium*, *R. nasisuis*, and *R. amarae*) found in pigs encoded multidrug-resistance gene elements (Figure 2), likely due to the strong selection pressure from the use of antibiotics for treatment of bacterial infections or use as growth promoters in livestock animals prior to the European ban in 2006 (Regulation (EC) No. 1831/2003). Specific tetracycline resistance genes were found in genomes of *Rothia* strains isolated from human *(tet*(W)) and porcine strains (*tet*(M)). In the past, the presence/absence of *tet* genes were mainly determined by sampling niche and application of tetracyclines, however, at present, *tet* genes have evolved and occurred more widely dispersed in different environments being present in commensal and pathogenic strains (59). Erythromycin is an antibiotic used to treat several respiratory infections and resistance genes (*erm*(X)), were present in human (n=27) and some porcine strains (n=7). However, a small number of antibiotic resistant genes were found in the *Rothia* genomes as a reflection of the continuous use in humans and animals on a large scale (60).

As different host organisms and host regions (i.e. tonsil or saliva) can contain different carbohydrates, it is of interest to characterise the carbon-converting enzymatic "toolbox" of *Rothia* strains sampled from different host organisms and environmental niches. The GH enzymes generally participate in carbohydrate catabolism, suggesting that the enzyme orthologs present in the *Rothia* genomes

have the potential to degrade complex carbohydrates originating from mammals, plants, insects, and fungi including mucins, cellulose, membrane glycoproteins, and chitin in acidic environments (61). The high number of GTs present in *Rothia* genomes are likely involved in carbohydrate biosynthesis, although some GTs have been reported to enzymatically attach carbohydrate moieties to precursors of some naturally occurring antibiotics (62, 63).

GTs identified in *Rothia* genomes can be responsible for the synthesis and assembly of the repetitive units of exopolysaccharides (EPS), which contributes for the biofilm formation, tolerance to desiccation and host antimicrobial peptides, as well as modulation the host immune response by the association with other molecules as glycolipids (64). The CAZy families GT2 and GT4 are present in all *Rothia* genomes and are involved in the biosynthesis of various (exo)polysaccharides, which are often involved in biofilm formation, and biosynthesis of natural products (65). Most *Rothia* isolates have a mucoid morphology (Supplementary table 1) and aggregate in liquid media which might be directly associated with the EPS production although the morphology of the colonies varies at the species level depending on the *in vitro* culture conditions. Exopolysaccharides could also provide alternative carbon sources to support the establishment of a pioneer microbial community in the upper respiratory tract (66). The genomes of strains from the species *R. dentocariosa* and *R. mucilaginosa,* which colonise the human oral cavity, contained the largest numbers of predicted GT2 genes (Figure 4). Considering exopolysaccharides are one of the main structural components of bacterial extracellular matrix and biofilm (67), the high amount of GTs may favour the formation of structurally organized biofilm consortia composed by a single taxon or a cluster of taxa interacting with *Rothia* on the oral mucosa and on teeth surfaces (2).

The CAZy families GH33, GH36, GT5, GT35, CBM48+GH13-11 and CBM48+GH13-9 (CAZy Group 1) were mostly present in the animal-associated species (*R. nasimurium* and *R. nasisuis*). The GH33 and GH36 families, are mainly present in the porcine isolates and are known to be involved in the utilisation of human milk oligosaccharides (HMOs) and O-glycosylated mucins. HMO's shape the microbiota and have beneficial effects in early life (68). The capacity to catabolise HMOs and glycosylated mucins may be an advantage to pioneer species and drive microbiota diversity (69). Several CAZy families contain enzymes involved in carbohydrate digestion and hydrolysation. CAZy family GT35 includes glycogen or starch phosphorylase enzymes, family CBM48 includes enzymes participating in carbohydrate binding and glycogen-binding, and GH13 includes enzymes with glycosyl hydrolase activity. Little information is available about the utilization of complex carbohydrates in the oral cavity, but it is well described that the enzymes that release energy from

the breakdown of branched substrates and complex carbohydrates can support proliferation and survival of certain species when there other carbon sources are limiting, and by maintaining microbiota diversity, have a positive impact on health (70).

The families GT25 and CBM50+GH23 contain enzymes with glycosyltransferase activity and are mostly present in the human-associated *Rothia* species. The CAZy family GT25 plays important roles in catalysing utilization of monosaccharides, assembly of glycoconjugates and complex carbohydrates by transferring sugar moieties onto growing liposaccharide chains (PF01755). GT25 enzymes may play a role in utilization of carbohydrates from different sources, and in biofilm formation, while the family complex CBM50+GH23 is associated with lytic transglycosylase activity related to the peptidoglycan metabolic process. Proteins with this annotation are usually enzymes active in the breakdown of chitin or peptidoglycan into molecules that tigger innate immune responses though recognition by host pattern recognition receptors (71).

The CAZY enzymes GH65, CBM50, and GT20 were only identified in *Rothia* species isolated from environmental niches. The GT20 family contains enzymes annotated with trehalose-phosphatase activity (PF00982) while the GH65 family contains acid trehalases and some phosphorylases that catalyse conversion of trehalose to glucose and glucose-1-phosphate. Trehalose is a common disaccharide used by bacteria, archaea, fungi, invertebrates and others as a carbon source but trehalose also plays an important role in protecting bacteria against stress including desiccation, osmotic stress, oxidation, and temperature changes (72). The CBM50 or LysM domain family members contain a domain of approximately 40-50 amino acid residues that can occur in various carbohydrate-modifying enzymes, for instance, glycoside hydrolase enzymes that play roles in the digestion of complex carbohydrates (73). The LysM domain is found in carbohydrate-active enzymes, peptidoglycan binding proteins and plant cell surface receptors for fungal chitin oligosaccharides (73, 74). The LysM domain is also found in proteins correlated with several other biological functions including stress response in plants (75), signalling for specific plant and bacteria interactions (76) and bacterial spore surface development (77). As these modules are involved in interactions between different organisms, they may play roles during symbiosis and quorum sensing in soil- or plant-associated bacteria during microbiota development. Many of the CAZy enzymes identified in *Rothia* correlate with the environmental or host niche, emphasising that these enzymes have important roles in microbial ecology and are relevant determinants of which habitats can be colonised and exploited by microbes. *Rothia* species encode CAZy genes for breakdown of chitin in fungal and insect cell walls, mucins and milk oligosaccharides into simple carbohydrates that

can be used as nutrients and carbon sources for fermentation by other microbiota members (79). Such cross-feeding might enable symbiotic interactions between *Rothia* and other microbes and could benefit microbial homeostasis and symbiosis from early life onward.

The presence of genes related to the transport, assimilation and conversion of nitrate to nitrite, denitrification, nitric oxide detoxification and bacterial nitrate reduction enzymes cofactor were identified in several species and highly correlated with the source of isolation (Supplementary Table 5). We noted that the human associated species *R. dentocariosa, R. mucilaginosa, R. aerea* and *Rothia* sp. contain most of genes involved in nitrate and nitrite metabolic pathways as reported previously in 10 human-associated oral *Rothia* species isolates (79). Porcine and other animal associated species as *R. nasimurium* and *R. nasisuis* also carry some of the genes involved in nitrate and nitrite metabolism, suggesting that those species might have similar function(s) in the oral cavity of animals, potentially with consequences for health and disease (32). We noted that environmental species as *R. kristinae* and *R. koreensis* appeared to lack most of nitrate/nitrite metabolic genes. Nitrate/nitrite metabolic genes await functional characterisation. This topic warrants further studies using physiological measurements of nitrate reduction for different *Rothia* species.

In summary, we showed that the NRPS producing the antimicrobial valinomycin was specific to *R. nasisuis* isolates from pig upper respiratory tract and identified novel BGCs encoding NRPSs, PKSs and RiPPs in other *Rothia* species that may produce antimicrobial compounds. According to our analyses, *Rothia* species have an extensive number of CAZy (5,040 genes annotated in total), many of which are associated with EPS production and catabolism of carbohydrate sources (possibly including milk oligosaccharides) in different hosts and environments. Members of the *Rothia* genus have potential to produce novel antimicrobials and may be used as probiotics to shape the microbiota of humans and mammals in early life and provide colonization resistance against pathogens and pathobionts.

List of Abbreviations

BGC, biosynthetic gene cluster; CAZY, carbohydrate-active enzymes; NRPS, non-ribosomal peptide synthetase; NRPs non-ribosomal peptides; RiPPs, post-translationally modified peptides, PKs, polyketides; ARGs, Antimicrobial resistance genes.

Data Summary

All datasets generated for this study are included in the manuscript and/or in the Supplementary Files. The whole-genome sequencing data is available at the European Nucleotide Archive (ENA) under BioProject ID PRJEB49523.

Ethical approval

This study uses samples obtained for diagnostic procedures performed according to the ethical principles and guidelines covered by EU Directive 2010/63/EU.

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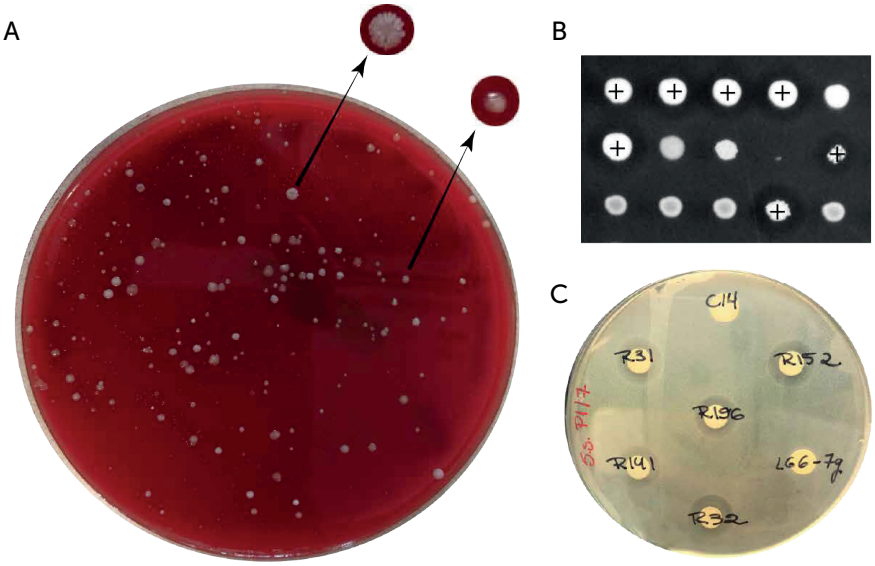
Supplementary material



Supplementary Figure 1. (A) Columbia agar plate containing bacterial colonies; insert showing two Rothia colonies selected on basis of colony morphology, **(B)** and **(C)** Screening overlay inhibition assays on agar plates to identify colonies inhibiting growth of target bacteria in the overlay and inhibition zones ("halos") where the growth of target bacteria is inhibited by products secreted by candidate antagonist.

Supplementary Table 1. Identification of the 112 genome sequences of publicly available *Rothia* species downloaded from NCBI's Reference Sequence database used in this study (download 27/04/2021) with the 43 porcine *Rothia* sp. isolates from this study.

Organism Scientific Name	ID	Isolation source	Sampling site	Taxonomy ID	Assembly Accession	Source
Rothia aeria	strain: C6B	human	sputum	172042	GCF_000763685.1	RefSeq
Rothia aeria	strain: C6D	human	sputum	172042	GCF_000763765.1	RefSeq
Rothia aeria	strain: FDAARGOS_1137	NA	Missing	172042	GCF_016726365.1	RefSeq
Rothia aeria	strain: NCTC10207	NA	Missing	172042	GCF_900637985.1	RefSeq
Rothia aeria F0184	strain: F0184	human	oral cavity	888019	GCF_000479025.1	RefSeq
Rothia aeria F0474	strain: F0474	human	Missing	1125724	GCF_000258205.1	RefSeq
Rothia aerolata	strain: CCM 8669	NA	Missing	1812262	GCF_014635585.1	RefSeq
Rothia amarae	strain: KJZ-9	environment	Dirt	169480	GCF_014705945.2	RefSeq
Rothia dentocariosa	strain: 1234_RDEN	human	Missing	2047	GCF_001061305.1	RefSeq
Rothia dentocariosa	strain: 1233_RDEN	human	Missing	2047	GCF_001062425.1	RefSeq
Rothia dentocariosa	strain: 316_RDEN	human	Missing	2047	GCF_001064485.1	RefSeq
Rothia dentocariosa	strain: 694_RDEN	human	Missing	2047	GCF_001066935.1	RefSeq
Rothia dentocariosa	strain: OG2-1	food	Kefir	2047	GCF_002276695.1	RefSeq
Rothia dentocariosa	strain: OG2-2	food	Kefir	2047	GCF_002554715.1	RefSeq
Rothia dentocariosa	strain: UMB0083	human	urine catheter	2047	GCF_002861025.1	RefSeq
Rothia dentocariosa	strain: 1C11	environment	Alder root nodule	2047	GCF_004563855.1	RefSeq
Rothia dentocariosa	strain: TB22-02	environment	Toothbrush	2047	GCF_012641675.1	RefSeq
Rothia dentocariosa	strain: FDAARGOS_752	human	sputum	2047	GCF_013267735.1	RefSeq
Rothia dentocariosa	strain: NCTC10917	human	Carious teeth	2047	GCF_900455895.1	RefSeq
Rothia dentocariosa	isolate: BgEED26	human	duodenal aspirate	2047	GCF_901875315.1	RefSeq
Rothia dentocariosa ATCC 17931	strain: ATCC 17931	human	teeth	762948	GCF_000164695.2	RefSeq
Rothia dentocariosa M567	strain: M567	human	airways	563032	GCF_000143585.1	RefSeq
Rothia halotolerans	strain: JCM 31975	environment	Saline soil	405770	GCF_004136635.1	RefSeq
Rothia koreensis	strain: JCM 15915	food	jeotgal	592378	GCF_004136575.1	RefSeq
Rothia koreensis	strain: JCM 15915	food	seafood	592378	GCF_009734705.1	RefSeq
Rothia kristinae	strain: RUTW2-3	Tsitsikamma	Missing	37923	GCF_001420025.2	RefSeq

Level	Contig N50	Size	Submission Date	Gene Count	BioProject	BioSample	Species
Contig	135891	2644345	6/10/2014	2340	PRJNA246623	SAMN02996612	*Rothia aeria*
Contig	200143	2639435	6/10/2014	2340	PRJNA246626	SAMN02954007	*Rothia aeria*
Complete Genome	2583917	2583917	20/01/2021	2267	PRJNA231221	SAMN16357306	*Rothia aeria*
Complete Genome	2707814	2707814	20/12/2018	2400	PRJEB6403	SAMEA103980422	*Rothia aeria*
Scaffold	81275	2603188	23/10/2013	2305	PRJNA52997	SAMN02436738	*Rothia aeria*
Contig	109779	2584293	6/4/2012	2259	PRJNA78909	SAMN00792226	*Rothia aeria*
Scaffold	533970	2462803	11/9/2020	2217	PRJDB10511	SAMD00244869	*Rothia aerolata*
Complete Genome	2407675	2483880	2/11/2020	2315	PRJNA663431	SAMN16132388	*Rothia amarae*
Scaffold	77838	2491306	10/7/2015	2157	PRJNA267549	SAMN03197199	*Rothia dentocariosa*
Contig	62525	2476244	10/7/2015	2146	PRJNA267549	SAMN03197198	*Rothia dentocariosa*
Scaffold	177405	2504054	10/7/2015	2178	PRJNA267549	SAMN03197506	*Rothia dentocariosa*
Contig	108748	2425579	10/7/2015	2110	PRJNA267549	SAMN03197898	*Rothia dentocariosa*
Contig	279534	2469903	29/08/2017	2142	PRJNA375758	SAMN06718484	*Rothia dentocariosa*
Contig	1241720	2446762	16/10/2017	2137	PRJNA375758	SAMN07759903	*Rothia dentocariosa*
Contig	816369	2494324	28/12/2017	2146	PRJNA316969	SAMN08193714	*Rothia dentocariosa*
Contig	51190	2536666	1/4/2019	2265	PRJNA480027	SAMN11249661	*Rothia dentocariosa*
Contig	160318	2597203	21/04/2020	2276	PRJNA559375	SAMN12537708	*Rothia dentocariosa*
Complete Genome	2533415	2533415	4/6/2020	2188	PRJNA231221	SAMN11056467	*Rothia dentocariosa*
Contig	2462898	2490333	1/8/2018	2146	PRJEB6403	SAMEA44521918	*Rothia dentocariosa*
Contig	323595	2453562	14/02/2021	2128	PRJEB32184	SAMEA5664370	*Rothia dentocariosa*
Complete Genome	2506025	2506025	3/11/2010	2150	PRJNA48447	SAMN00120582	*Rothia dentocariosa*
Scaffold	234454	2532787	9/7/2010	2199	PRJNA42539	SAMN02463840	*Rothia dentocariosa*
Contig	1054027	3037640	4/2/2019	2751	PRJNA393544	SAMN07337062	*Rothia halotolerans*
Contig	118248	2911929	4/2/2019	2607	PRJNA393544	SAMN07337066	*Rothia koreensis*
Scaffold	58277	2929181	8/12/2019	2619	PRJNA591111	SAMN13352282	*Rothia koreensis*
Contig	99764	2356130	8/6/2016	2109	PRJNA294377	SAMN04025229	*Rothia kristinae*

Supplementary Table 1. Continued.

Organism Scientific Name	ID	Isolation source	Sampling site	Taxonomy ID	Assembly Accession	Source
Rothia kristinae	strain: SA12	environment	Oryza sativa	37923	GCF_001475915.1	RefSeq
Rothia kristinae	strain: RSA5	environment	Oryza sativa	37923	GCF_001476145.1	RefSeq
Rothia kristinae	strain: SA11	environment	Oryza sativa	37923	GCF_001476175.1	RefSeq
Rothia kristinae	strain: RSA28	environment	Oryza sativa	37923	GCF_001476195.1	RefSeq
Rothia kristinae	strain: SA15	environment	Oryza sativa	37923	GCF_001476205.1	RefSeq
Rothia kristinae	strain: SA13	environment	Oryza sativa	37923	GCF_001476995.1	RefSeq
Rothia kristinae	strain: SA14	environment	Oryza sativa	37923	GCF_001477035.1	RefSeq
Rothia kristinae	strain: LCT-H5	environment	Oryza sativa	37923	GCF_001866115.1	RefSeq
Rothia kristinae	strain: ATCC 27570	human	skin	37923	GCF_004136565.1	RefSeq
Rothia kristinae	strain: DE0322	environment	Missing	37923	GCF_007681115.1	RefSeq
Rothia kristinae	strain: GM	environment	Missing	37923	GCF_015765105.1	RefSeq
Rothia kristinae	strain: FDAARGOS_864	NA	Missing	37923	GCF_016028855.1	RefSeq
Rothia kristinae	strain: FDAARGOS_1001	NA	Missing	37923	GCF_016128175.1	RefSeq
Rothia kristinae NBRC 15354	strain: NBRC 15354	NA	Missing	1349768	GCF_001570865.1	RefSeq
Rothia mucilaginosa	strain: 1211_RMUC	human	clinical or host-associated	43675	GCF_001060545.1	RefSeq
Rothia mucilaginosa	strain: 138_RMUC	human	clinical or host-associated	43675	GCF_001061655.1	RefSeq
Rothia mucilaginosa	strain: 141_RMUC	human	clinical or host-associated	43675	GCF_001061665.1	RefSeq
Rothia mucilaginosa	strain: 175_RMUC	human	clinical or host-associated	43675	GCF_001062855.1	RefSeq
Rothia mucilaginosa	strain: 509_RMUC	human	clinical or host-associated	43675	GCF_001063545.1	RefSeq
Rothia mucilaginosa	strain: 207_RMUC	human	clinical or host-associated	43675	GCF_001063995.1	RefSeq
Rothia mucilaginosa	strain: 268_RMUC	human	clinical or host-associated	43675	GCF_001064265.1	RefSeq

Level	Contig N50	Size	Submission Date	Gene Count	BioProject	BioSample	Species
Contig	17257	2215904	22/12/2015	2061	PRJNA278411	SAMN03401335	*Rothia kristinae*
Contig	21282	2280770	22/12/2015	2095	PRJNA278175	SAMN03401339	*Rothia kristinae*
Contig	19601	2242755	22/12/2015	2063	PRJNA278410	SAMN03401334	*Rothia kristinae*
Contig	25292	2296741	22/12/2015	2091	PRJNA278176	SAMN03401340	*Rothia kristinae*
Contig	19130	2265036	22/12/2015	2083	PRJNA278414	SAMN03401338	*Rothia kristinae*
Contig	15889	2251567	22/12/2015	2097	PRJNA278412	SAMN03401336	*Rothia kristinae*
Contig	22988	2216948	22/12/2015	2024	PRJNA278413	SAMN03401337	*Rothia kristinae*
Scaffold	188369	2409488	3/11/2016	2149	PRJNA350375	SAMN05938613	*Rothia kristinae*
Contig	419088	2362630	4/2/2019	2098	PRJNA393544	SAMN07337061	*Rothia kristinae*
Scaffold	161583	2559995	30/07/2019	2336	PRJNA543692	SAMN11792482	*Rothia kristinae*
Contig	94865	2373046	4/12/2020	2142	PRJNA679472	SAMN16837239	*Rothia kristinae*
Complete Genome	2377741	2377741	14/12/2020	2106	PRJNA231221	SAMN13450394	*Rothia kristinae*
Complete Genome	2513835	2513835	20/12/2020	2222	PRJNA231221	SAMN16357170	*Rothia kristinae*
Contig	137365	2355726	10/2/2016	2109	PRJDB1352	SAMD00046484	*Rothia kristinae*
Scaffold	154121	2277731	10/7/2015	1808	PRJNA267549	SAMN03197174	*Rothia mucilaginosa*
Scaffold	103793	2308239	10/7/2015	1817	PRJNA267549	SAMN03197327	*Rothia mucilaginosa*
Scaffold	204474	2236745	10/7/2015	1805	PRJNA267549	SAMN03197331	*Rothia mucilaginosa*
Scaffold	207965	2292653	10/7/2015	1804	PRJNA267549	SAMN03197366	*Rothia mucilaginosa*
Scaffold	98433	2374318	10/7/2015	1883	PRJNA267549	SAMN03197702	*Rothia mucilaginosa*
Scaffold	227095	2323428	10/7/2015	1829	PRJNA267549	SAMN03197400	*Rothia mucilaginosa*
Scaffold	263636	2275643	10/7/2015	1808	PRJNA267549	SAMN03197461	*Rothia mucilaginosa*

Supplementary Table 1. Continued.

Organism Scientific Name	ID	Isolation source	Sampling site	Taxonomy ID	Assembly Accession	Source
Rothia mucilaginosa	strain: 328_RMUC	human	clinical or host-associated	43675	GCF_001064575.1	RefSeq
Rothia mucilaginosa	strain: 329_RMUC	human	clinical or host-associated	43675	GCF_001064585.1	RefSeq
Rothia mucilaginosa	strain: 470_RMUC	human	clinical or host-associated	43675	GCF_001065115.1	RefSeq
Rothia mucilaginosa	strain: 473_RMUC	human	clinical or host-associated	43675	GCF_001065135.1	RefSeq
Rothia mucilaginosa	strain: 574_RMUC	human	clinical or host-associated	43675	GCF_001065485.1	RefSeq
Rothia mucilaginosa	strain: 788_RMUC	human	clinical or host-associated	43675	GCF_001067345.1	RefSeq
Rothia mucilaginosa	strain: 902_RMUC	human	clinical or host-associated	43675	GCF_001067855.1	RefSeq
Rothia mucilaginosa	strain: NUM-Rm6536	human	teeth plaque	43675	GCF_001548235.1	RefSeq
Rothia mucilaginosa	strain: FDAARGOS_369	human	blood	43675	GCF_002386365.1	RefSeq
Rothia mucilaginosa	strain: UMB0024	human	urine catheter	43675	GCF_002861015.1	RefSeq
Rothia mucilaginosa	strain: DE0531	environment	Missing	43675	GCF_007666515.1	RefSeq
Rothia mucilaginosa	strain: CECT30005	human	tongue	43675	GCF_015240415.1	RefSeq
Rothia mucilaginosa	isolate: BgEED27	human	duodenal aspirate	43675	GCF_901875305.1	RefSeq
Rothia mucilaginosa ATCC 25296	strain: ATCC 25296	human	Pharyngeal mucosa	553201	GCF_000175615.1	RefSeq
Rothia mucilaginosa DY-18	strain: DY-18	NA	Missing	680646	GCF_000011025.1	RefSeq
Rothia mucilaginosa M508	strain: M508	human	airways	563033	GCF_000231235.1	RefSeq
Rothia nasimurium	strain: PT-32	Porcine	Tonsil swab	85336	GCF_002087015.1	RefSeq
Rothia nasimurium	strain: irhom_31	animal	Oral swab	85336	GCF_004569295.1	RefSeq

Level	Contig N50	Size	Submission Date	Gene Count	BioProject	BioSample	Species
Scaffold	23185	2337099	10/7/2015	1926	PRJNA267549	SAMN03197517	*Rothia mucilaginosa*
Contig	72424	2359913	10/7/2015	1888	PRJNA267549	SAMN03197518	*Rothia mucilaginosa*
Scaffold	186368	2244809	10/7/2015	1760	PRJNA267549	SAMN03197665	*Rothia mucilaginosa*
Scaffold	75081	2271708	10/7/2015	1832	PRJNA267549	SAMN03197666	*Rothia mucilaginosa*
Scaffold	31877	2232412	10/7/2015	1808	PRJNA267549	SAMN03197777	*Rothia mucilaginosa*
Contig	20253	2257570	10/7/2015	1853	PRJNA267549	SAMN03197999	*Rothia mucilaginosa*
Contig	90270	2264347	10/7/2015	1784	PRJNA267549	SAMN03198110	*Rothia mucilaginosa*
Complete Genome	2292716	2292716	12/8/2015	1789	PRJDB4139	SAMD00036190	*Rothia mucilaginosa*
Complete Genome	2369230	2408770	28/09/2017	1912	PRJNA231221	SAMN07312413	*Rothia mucilaginosa*
Scaffold	553330	2258154	28/12/2017	1782	PRJNA316969	SAMN08193715	*Rothia mucilaginosa*
Contig	144164	2398511	30/07/2019	2270	PRJNA543692	SAMN11792691	*Rothia mucilaginosa*
Chromosome	2316333	2316433	5/11/2020	1814	PRJNA658327	SAMN15866159	*Rothia mucilaginosa*
Contig	349170	2236411	14/02/2021	1791	PRJEB32184	SAMEA5664371	*Rothia mucilaginosa*
Contig	172461	2255158	5/8/2009	1784	PRJNA31405	SAMN00001919	*Rothia mucilaginosa*
Complete Genome	2264603	2264603	5/1/2010	1768	PRJDA38547	SAMD00060968	*Rothia mucilaginosa*
Scaffold	277776	2313271	21/10/2011	1836	PRJNA38789	SAMN02463763	*Rothia mucilaginosa*
Contig	213550	2678677	11/4/2017	2408	PRJNA321506	SAMN04998822	*Rothia nasisuis*
Contig	51979	2688339	2/4/2019	2597	PRJNA527079	SAMN11126867	*Rothia nasimurium*

Supplementary Table 1. Continued.

Organism Scientific Name	ID	Isolation source	Sampling site	Taxonomy ID	Assembly Accession	Source
Rothia nasimurium	strain: E1706032	animal	Brain	85336	GCF_014217215.1	RefSeq
Rothia nasimurium	strain: 19428wA5_ irhom_31	animal	Oral swab	85336	GCF_015235405.1	RefSeq
Rothia sp. HMSC036D11	strain: HMSC036D11	human	bronch wash	1739462	GCF_001814205.1	RefSeq
Rothia sp. HMSC058E10	strain: HMSC058E10	human	bronchoalveolar lavage	1715088	GCF_001809415.1	RefSeq
Rothia sp. HMSC061C12	strain: HMSC061C12	human	throat	1739547	GCF_001815755.1	RefSeq
Rothia sp. HMSC061D12	strain: HMSC061D12	human	sputum	1715161	GCF_001836735.1	RefSeq
Rothia sp. HMSC061E04	strain: HMSC061E04	human	sputum	1739431	GCF_001813685.1	RefSeq
Rothia sp. HMSC062F03	strain: HMSC062F03	human	sputum	1715153	GCF_001836905.1	RefSeq
Rothia sp. HMSC062H08	strain: HMSC062H08	human	sputum	1739269	GCF_001810775.1	RefSeq
Rothia sp. HMSC064D08	strain: HMSC064D08	human	sputum	1715104	GCF_001809525.1	RefSeq
Rothia sp. HMSC064F07	strain: HMSC064F07	human	sputum	1715191	GCF_001837265.1	RefSeq
Rothia sp. HMSC065B04	strain: HMSC065B04	human	sputum	1739349	GCF_001812285.1	RefSeq
Rothia sp. HMSC065C03	strain: HMSC065C03	human	sputum	1715084	GCF_001837295.1	RefSeq
Rothia sp. HMSC065C12	strain: HMSC065C12	human	sputum induced	1739340	GCF_001812085.1	RefSeq
Rothia sp. HMSC065D02	strain: HMSC065D02	human	sputum induced	1739518	GCF_001815285.1	RefSeq
Rothia sp. HMSC065D09	strain: HMSC065D09	human	Bronchoalveolar lavage	1739511	GCF_001815155.1	RefSeq
Rothia sp. HMSC065G12	strain: HMSC065G12	human	sputum induced	1739308	GCF_001811455.1	RefSeq
Rothia sp. HMSC066G02	strain: HMSC066G02	human	sputum	1739398	GCF_001813145.1	RefSeq
Rothia sp. HMSC066G07	strain: HMSC066G07	human	sputum	1739475	GCF_001814485.1	RefSeq

Level	Contig N50	Size	Submission Date	Gene Count	BioProject	BioSample	Species
Complete Genome	2443067	2526221	17/08/2020	2301	PRJNA639408	SAMN15235892	*Rothia nasimurium*
Contig	51979	2688339	4/11/2020	2582	PRJNA671681	SAMN16540187	*Rothia nasimurium*
Scaffold	156199	2491181	21/10/2016	2140	PRJNA300112	SAMN04480381	*Rothia sp*
Scaffold	141745	2439224	21/10/2016	2123	PRJNA296216	SAMN04477506	*Rothia sp*
Scaffold	48292	2282454	21/10/2016	1819	PRJNA300198	SAMN04480464	*Rothia sp*
Scaffold	104363	2336535	25/10/2016	1846	PRJNA296289	SAMN04498645	*Rothia sp*
Scaffold	122101	2237336	21/10/2016	1773	PRJNA300081	SAMN04480354	*Rothia sp*
Scaffold	75670	2271942	25/10/2016	1816	PRJNA296281	SAMN04498642	*Rothia sp*
Scaffold	65159	2299565	21/10/2016	1815	PRJNA299919	SAMN04477583	*Rothia sp*
Scaffold	122905	2459656	21/10/2016	2131	PRJNA296232	SAMN04477514	*Rothia sp*
Scaffold	169082	2267615	25/10/2016	1787	PRJNA296319	SAMN04498655	*Rothia sp*
Scaffold	93931	2319336	21/10/2016	1827	PRJNA299999	SAMN04477657	*Rothia sp*
Scaffold	92136	2352774	25/10/2016	1860	PRJNA296212	SAMN04498657	*Rothia sp*
Scaffold	134628	2274293	21/10/2016	1822	PRJNA299990	SAMN04477648	*Rothia sp*
Scaffold	83304	2478701	21/10/2016	2145	PRJNA300169	SAMN04480436	*Rothia sp*
Scaffold	91541	2331353	21/10/2016	1869	PRJNA300161	SAMN04480428	*Rothia sp*
Scaffold	84257	2512257	21/10/2016	2157	PRJNA299958	SAMN04477617	*Rothia sp*
Scaffold	80971	2269254	21/10/2016	1793	PRJNA300048	SAMN04480060	*Rothia sp*
Scaffold	77840	2298993	21/10/2016	1822	PRJNA300125	SAMN04480395	*Rothia sp*

Supplementary Table 1. Continued.

Organism Scientific Name	ID	Isolation source	Sampling site	Taxonomy ID	Assembly Accession	Source
Rothia sp. HMSC066H02	strain: HMSC066H02	human	bronchial washing	1739503	GCF_001814995.1	RefSeq
Rothia sp. HMSC067H10	strain: HMSC067H10	human	bronchial washing	1739260	GCF_001810635.1	RefSeq
Rothia sp. HMSC068E02	strain: HMSC068E02	human	nasal	1739423	GCF_001813565.1	RefSeq
Rothia sp. HMSC068F09	strain: HMSC068F09	human	sputum	1739378	GCF_001812775.1	RefSeq
Rothia sp. HMSC069C01	strain: HMSC069C01	human	sputum	1739485	GCF_001814655.1	RefSeq
Rothia sp. HMSC069C03	strain: HMSC069C03	human	sputum	1739283	GCF_001811075.1	RefSeq
Rothia sp. HMSC069C04	strain: HMSC069C04	human	sputum	1739383	GCF_001812895.1	RefSeq
Rothia sp. HMSC069C10	strain: HMSC069C10	human	sputum	1739346	GCF_001812205.1	RefSeq
Rothia sp. HMSC069D01	strain: HMSC069D01	human	sputum induced	1715189	GCF_001810135.1	RefSeq
Rothia sp. HMSC071B01	strain: HMSC071B01	human	tracheal aspirate	1715007	GCF_001808945.1	RefSeq
Rothia sp. HMSC071C12	strain: HMSC071C12	human	tracheal aspirate	1739446	GCF_001813895.1	RefSeq
Rothia sp. HMSC071F11	strain: HMSC071F11	human	sputum	1715034	GCF_001809115.1	RefSeq
Rothia sp. HMSC072B03	strain: HMSC072B03	human	sputum induced	1715109	GCF_001809565.1	RefSeq
Rothia sp. HMSC072B04	strain: HMSC072B04	human	sputum	1739432	GCF_001813725.1	RefSeq
Rothia sp. HMSC072E10	strain: HMSC072E10	human	sputum	1739448	GCF_001813965.1	RefSeq
Rothia sp. HMSC073B08	strain: HMSC073B08	human	sputum	1739388	GCF_001812975.1	RefSeq
Rothia sp. HMSC075F09	strain: HMSC075F09	human	throat	1739253	GCF_001810515.1	RefSeq
Rothia sp. HMSC076D04	strain: HMSC076D04	human	sputum	1739484	GCF_001814645.1	RefSeq
Rothia sp. HMSC078H08	strain: HMSC078H08	human	tracheal aspirate	1715008	GCF_001808955.1	RefSeq

Level	Contig N50	Size	Submission Date	Gene Count	BioProject	BioSample	Species
Scaffold	129925	2331455	21/10/2016	1857	PRJNA300153	SAMN04480419	*Rothia sp*
Scaffold	117175	2490080	21/10/2016	2193	PRJNA299910	SAMN04477573	*Rothia sp*
Scaffold	89826	2296639	21/10/2016	1804	PRJNA300073	SAMN04480346	*Rothia sp*
Scaffold	60544	2239545	21/10/2016	1808	PRJNA300028	SAMN04480042	*Rothia sp*
Scaffold	106960	2416608	21/10/2016	2119	PRJNA300135	SAMN04480453	*Rothia sp*
Scaffold	75817	2217530	17/10/2016	1773	PRJNA299933	SAMN04477597	*Rothia sp*
Scaffold	76655	2314459	21/10/2016	1838	PRJNA300033	SAMN04480055	*Rothia sp*
Scaffold	62208	2285914	21/10/2016	1822	PRJNA299996	SAMN04477654	*Rothia sp*
Scaffold	69071	2346201	21/10/2016	1848	PRJNA296317	SAMN04477547	*Rothia sp*
Scaffold	102390	2421074	21/10/2016	1911	PRJNA296134	SAMN04456732	*Rothia sp*
Scaffold	103608	2353068	21/10/2016	1851	PRJNA300096	SAMN04480369	*Rothia sp*
Scaffold	79615	2547863	21/10/2016	2256	PRJNA296161	SAMN04495786	*Rothia sp*
Scaffold	61093	2235828	21/10/2016	1792	PRJNA296237	SAMN04477516	*Rothia sp*
Scaffold	85860	2269450	21/10/2016	1799	PRJNA300082	SAMN04480359	*Rothia sp*
Scaffold	59687	2245099	21/10/2016	1793	PRJNA300098	SAMN04480370	*Rothia sp*
Scaffold	94603	2272208	21/10/2016	1802	PRJNA300038	SAMN04480051	*Rothia sp*
Scaffold	74420	2309498	21/10/2016	1816	PRJNA299903	SAMN04477587	*Rothia sp*
Scaffold	71050	2321240	21/10/2016	1854	PRJNA300134	SAMN04480404	*Rothia sp*
Scaffold	79323	2312383	21/10/2016	1803	PRJNA296135	SAMN04456733	*Rothia sp*

Supplementary Table 1. Continued.

Organism Scientific Name	ID	Isolation source	Sampling site	Taxonomy ID	Assembly Accession	Source
Rothia sp. HMSC08A08	strain: HMSC08A08	human	sputum	1581132	GCF_001808385.1	RefSeq
Rothia sp. HSID18067	strain: HSID18067	human	nasal lavage	2419514	GCF_003989445.1	RefSeq
Rothia sp. HSID18069	strain: HSID18069	human	nasal lavage	2419515	GCF_003989155.1	RefSeq
Rothia sp. ND6WE1A	strain: ND6WE1A	environment	seawater	1848190	GCF_001683935.1	RefSeq
Rothia sp. Olga	strain: Olga	food	Kefir	1979525	GCF_002238135.1	RefSeq
Rothia sp. ZJ1223	strain: ZJ1223	animal	Missing	2811098	GCF_016900985.1	RefSeq
Rothia sp. ZJ932	strain: ZJ932	animal	Missing	2810516	GCF_016924835.1	RefSeq
Rothia terrae	strain: LMG 23708	environment	seawater	396015	GCF_012396615.1	RefSeq
Rothia terrae	strain: KJZ-14	environment	Dirt	396015	GCF_014705925.2	RefSeq
Rothia nasisuis 107RC1	107RC1	Porcine	Tonsil swab	2109647	ERS9600914	
Rothia nasimurium 110RC1	110RC1	Porcine	Tonsil swab	85336	ERS9600909	
Rothia nasimurium 123RC1	123RC1	Porcine	Tonsil swab	85336	ERS9600878	
Rothia nasimurium 124RC1	124RC1	Porcine	Tonsil swab	85336	ERS9600879	
Rothia nasimurium 136RC1	136RC1	Porcine	Tonsil swab	85336	ERS9600910	
Rothia nasisuis 141RC1	141RC1	Porcine	Tonsil swab	2109647	ERS9600880	
Rothia nasisuis 152RC1	152RC1	Porcine	Tonsil swab	2109647	ERS9600881	
Rothia aerolata 159RC1	159RC1	Porcine	Tonsil swab	85336	ERS9600882	
Rothia nasimurium 15QC2CO	15QC2CO	Porcine	Tonsil swab	85336	ERS9600894	
Rothia nasimurium 15QC4O2	15QC4O2	Porcine	Tonsil swab	85336	ERS9600912	
Rothia nasimurium 18QC4O2	18QC4O2	Porcine	Tonsil swab	85336	ERS9600913	
Rothia nasisuis 196RC1	196RC1	Porcine	Tonsil swab	2109647	ERS9600883	
Rothia nasimurium 19QC2O2	19QC2O2	Porcine	Tonsil swab	85336	ERS9600887	

Level	Contig N50	Size	Submission Date	Gene Count	BioProject	BioSample	Species
Scaffold	83625	2486809	21/10/2016	2174	PRJNA269901	SAMN03434238	*Rothia sp*
Contig	832921	2641641	31/12/2018	2331	PRJNA492917	SAMN10118516	*Rothia sp*
Contig	1351879	2639394	31/12/2018	2301	PRJNA492917	SAMN10118517	*Rothia sp*
Contig	76299	2315165	12/7/2016	2159	PRJNA244670	SAMN04933465	*Rothia sp*
Contig	342298	2756010	1/8/2017	2486	PRJNA375758	SAMN06718477	*Rothia sp*
Scaffold	328906	2173177	16/02/2021	1983	PRJNA700476	SAMN17832333	*Rothia sp*
Complete Genome	2261372	2264101	24/02/2021	2064	PRJNA700477	SAMN17832352	*Rothia sp*
Contig	122067	2482397	15/04/2020	2251	PRJNA622446	SAMN14517838	*Rothia terrae*
Complete Genome	2479856	2660019	2/11/2020	2426	PRJNA663434	SAMN16132433	*Rothia terrae*
Contig			2021			SAMEA11953817	*Rothia nasisuis*
Contig			2021			SAMEA11953812	*Rothia nasimurium*
Contig			2021			SAMEA11953781	*Rothia nasimurium*
Contig			2021			SAMEA11953782	*Rothia nasimurium*
Contig			2021			SAMEA11953813	*Rothia nasimurium*
Contig			2021			SAMEA11953783	*Rothia nasisuis*
Contig			2021			SAMEA11953784	*Rothia nasisuis*
Contig			2021			SAMEA11953785	*Rothia aerolata*
Contig			2021			SAMEA11953797	*Rothia nasimurium*
Contig			2021			SAMEA11953815	*Rothia nasimurium*
Contig			2021			SAMEA11953816	*Rothia nasimurium*
Contig			2021			SAMEA11953786	*Rothia nasisuis*
Contig			2021			SAMEA11953790	*Rothia nasimurium*

Supplementary Table 1. Continued.

Organism Scientific Name	ID	Isolation source	Sampling site	Taxonomy ID	Assembly Accession	Source
Rothia nasimurium 206RC1	206RC1	Porcine	Tonsil swab	85336	ERS9600884	
Rothia nasimurium 207RC1	207RC1	Porcine	Tonsil swab	85336	ERS9600911	
Rothia nasimurium 213RC1	213RC1	Porcine	Tonsil swab	85336	ERS9600885	
Rothia nasimurium 22QC2CO	22QC2CO	Porcine	Tonsil swab	85336	ERS9600895	
Rothia nasimurium 28QC2CO	28QC2CO	Porcine	Tonsil swab	85336	ERS9600888	
Rothia nasimurium 2QC2O2	2QC2O2	Porcine	Tonsil swab	85336	ERS9600886	
Rothia nasisuis 31RC1	31RC1	Porcine	Tonsil swab	2109647	ERS9600872	
Rothia nasimurium 34QC2O2	34QC2O2	Porcine	Tonsil swab	85336	ERS9600896	
Rothia nasimurium 36QC2O2	36QC2O2	Porcine	Tonsil swab	85336	ERS9600897	
Rothia nasimurium 37QC2O2	37QC2O2	Porcine	Tonsil swab	85336	ERS9600898	
Rothia nasimurium 38QC2CO	38QC2CO	Porcine	Tonsil swab	85336	ERS9600899	
Rothia nasimurium 3QC2O2	3QC2O2	Porcine	Tonsil swab	85336	ERS9600889	
Rothia nasimurium 46QC2O2	46QC2O2	Porcine	Tonsil swab	85336	ERS9600900	
Rothia nasimurium 48QC2CO	48QC2CO	Porcine	Tonsil swab	85336	ERS9600901	
Rothia nasimurium 4QC2O2	4QC2O2	Porcine	Tonsil swab	85336	ERS9600890	
Rothia nasimurium 54QC2CO	54QC2CO	Porcine	Tonsil swab	85336	ERS9600902	
Rothia endophytica 56QC2O2	56QC2O2	Porcine	Tonsil swab	1324766	ERS9600903	
Rothia nasimurium 63QC2CO	63QC2CO	Porcine	Tonsil swab	85336	ERS9600904	
Rothia nasimurium 63RC1	63RC1	Porcine	Tonsil swab	85336	ERS9600873	

Level	Contig N50	Size	Submission Date	Gene Count	BioProject	BioSample	Species
Contig			2021			SAMEA11953787	*Rothia nasimurium*
Contig			2021			SAMEA11953814	*Rothia nasimurium*
Contig			2021			SAMEA11953788	*Rothia nasimurium*
Contig			2021			SAMEA11953798	*Rothia nasimurium*
Contig			2021			SAMEA11953791	*Rothia nasimurium*
Contig			2021			SAMEA11953789	*Rothia nasimurium*
Contig			2021			SAMEA11953775	*Rothia nasisuis*
Contig			2021			SAMEA11953799	*Rothia nasimurium*
Contig			2021			SAMEA11953800	*Rothia nasimurium*
Contig			2021			SAMEA11953801	*Rothia nasimurium*
Contig			2021			SAMEA11953802	*Rothia nasimurium*
Contig			2021			SAMEA11953792	*Rothia nasimurium*
Contig			2021			SAMEA11953803	*Rothia nasimurium*
Contig			2021			SAMEA11953804	*Rothia nasimurium*
Contig			2021			SAMEA11953793	*Rothia nasimurium*
Contig			2021			SAMEA11953805	*Rothia nasimurium*
Contig			2021			SAMEA11953806	*Rothia endophytica*
Contig			2021			SAMEA11953807	*Rothia nasimurium*
Contig			2021			SAMEA11953776	*Rothia nasimurium*

Supplementary Table 1. Continued.

Organism Scientific Name	ID	Isolation source	Sampling site	Taxonomy ID	Assembly Accession	Source
Rothia nasimurium 64QC2CO	64QC2CO	Porcine	Tonsil swab	85336	ERS9600905	
Rothia nasimurium 65.2RC1	65.2RC1	Porcine	Tonsil swab	85336	ERS9600874	
Rothia nasimurium 65RC1	65RC1	Porcine	Tonsil swab	85336	ERS9600906	
Rothia nasimurium 66RC1	66RC1	Porcine	Tonsil swab	85336	ERS9600875	
Rothia nasimurium 67.3RC1	67.3RC1	Porcine	Tonsil swab	85336	ERS9600907	
Rothia nasimurium 67RC1	67RC1	Porcine	Tonsil swab	85336	ERS9600876	
Rothia nasimurium 68RC1	68RC1	Porcine	Tonsil swab	85336	ERS9600908	
Rothia nasisuis 69RC1	69RC1	Porcine	Tonsil swab	2109647	ERS9600877	
Rothia nasimurium 6QC2O2	6QC2O2	Porcine	Tonsil swab	85336	ERS9600891	
Rothia nasimurium 7QC2O2	7QC2O2	Porcine	Tonsil swab	85336	ERS9600892	
Rothia nasimurium 8QC2CO	8QC2CO	Porcine	Tonsil swab	85336	ERS9600893	

Level	Contig N50	Size	Submission Date	Gene Count	BioProject	BioSample	Species
Contig			2021			SAMEA11953808	*Rothia nasimurium*
Contig			2021			SAMEA11953777	*Rothia nasimurium*
Contig			2021			SAMEA11953809	*Rothia nasimurium*
Contig			2021			SAMEA11953778	*Rothia nasimurium*
Contig			2021			SAMEA11953810	*Rothia nasimurium*
Contig			2021			SAMEA11953779	*Rothia nasimurium*
Contig			2021			SAMEA11953811	*Rothia nasimurium*
Contig			2021			SAMEA11953780	*Rothia nasisuis*
Contig			2021			SAMEA11953794	*Rothia nasimurium*
Contig			2021			SAMEA11953795	*Rothia nasimurium*
Contig			2021			SAMEA11953796	*Rothia nasimurium*

Supplementary Table 2. Core genes (n=28) determined based on 90% protein similarity.

Core genes	Annotation
sufC	Vegetative protein 296
purA	Adenylosuccinate synthetase
rpsE	hypothetical protein
rplU	50S ribosomal protein L21
group_1444	hypothetical protein
rpmD	50S ribosomal protein L30
rpsC	30S ribosomal protein S3
ptsH	Phosphocarrier protein HPr
tuf	Elongation factor Tu
rplP	50S ribosomal protein L16
rplB	50S ribosomal protein L2
rpmB	50S ribosomal protein L28
rpsN	30S ribosomal protein S14
rpmA	50S ribosomal protein L27
group_2261	hypothetical protein
rplT	50S ribosomal protein L20
rpsM	30S ribosomal protein S13
rpsK	30S ribosomal protein S11
rpsG	30S ribosomal protein S7
rpmJ	50S ribosomal protein L36
rplN	50S ribosomal protein L14
rpsS	30S ribosomal protein S19
rpmG2	50S ribosomal protein L33 2
rpmI	50S ribosomal protein L35
infA	Translation initiation factor IF-1
group_5621	hypothetical protein
rpsL	30S ribosomal protein S12
erpA	Protein

Supplementary Table 3. Genomic assemblies information of the 43 porcine *Rothia* sp. isolates.

Lab_ID	Species	Total length (kbp)	# contigs	GC (%)	N50
107RC1	*Rothia nasimurium*	2.68	190	58	26281
110RC1	*Rothia nasimurium*	2.43	30	60.15	231593
123RC1	*Rothia nasimurium*	2.41	71	60.23	54644
124RC1	*Rothia nasimurium*	2.43	173	60.04	22169
136RC1	*Rothia nasimurium*	2.41	24	60.17	247079
141RC1	*Rothia nasisuis*	2.77	50	57.88	131046
152RC1	*Rothia nasisuis*	2.73	26	57.95	315715
159RC1	*Rothia aerolata*	2.47	22	58	199803
15QC2CO	*Rothia nasimurium*	2.55	27	59.5	222666
15QC4O2	*Rothia nasimurium*	2.52	304	59.48	14136
18QC4O2	*Rothia nasimurium*	2.55	313	59.17	13066
196RC1	*Rothia nasisuis*	2.79	68	57.8	84267
19QC2O2	*Rothia nasimurium*	2.53	46	59.32	108327
206RC1	*Rothia nasimurium*	2.38	211	60.15	20472
207RC1	*Rothia nasimurium*	2.39	27	60.2	148595
213RC1	*Rothia nasimurium*	2.42	68	59.66	69969
22QC2CO	*Rothia nasimurium*	2.58	28	59.46	283646
28QC2CO	*Rothia nasimurium*	2.51	35	59.37	331108
2QC2O2	*Rothia nasimurium*	2.52	32	59.44	238984
31RC1	*Rothia nasisuis*	2.73	46	57.99	122172
34QC2O2	*Rothia nasimurium*	2.41	24	60.02	262566
36QC2O2	*Rothia nasimurium*	2.46	27	59.6	196172
37QC2O2	*Rothia nasimurium*	2.41	24	59.64	255161
38QC2CO	*Rothia nasimurium*	2.46	28	59.45	177622
3QC2O2	*Rothia nasimurium*	2.41	28	60.02	184158
46QC2O2	*Rothia nasimurium*	2.49	29	59.55	175522
48QC2CO	*Rothia nasimurium*	2.56	25	59.49	283508
4QC2O2	*Rothia nasimurium*	2.42	30	59.62	213882
54QC2CO	*Rothia nasimurium*	2.49	30	59.55	260502
56QC2O2	*Rothia endophytica*	2.61	33	56.68	334637
63QC2CO	*Rothia nasimurium*	2.56	33	59.5	173856
63RC1	*Rothia nasimurium*	2.44	41	59.66	132630
64QC2CO	*Rothia nasimurium*	2.59	33	59.47	176843

Supplementary Table 3. Continued.

Lab_ID	Species	Total length (kbp)	# contigs	GC (%)	N50
652RC1	*Rothia nasimurium*	2.48	147	59.95	29021
65RC1	*Rothia nasimurium*	2.47	34	59.98	182993
66RC1	*Rothia nasimurium*	2.41	84	60.16	47114
673RC1	*Rothia nasimurium*	2.43	29	60.08	245080
67RC1	*Rothia nasimurium*	2.48	117	59.96	32327
68RC1	*Rothia nasimurium*	2.41	20	60.24	229402
69RC1	*Rothia nasisuis*	2.71	38	57.99	170701
6QC2O2	*Rothia nasimurium*	2.42	31	59.62	213882
7QC2O2	*Rothia nasimurium*	2.56	39	59.5	134360
8QC2CO	*Rothia nasimurium*	2.49	32	59.37	412686

Supplementary Table 4. Total number of Carbohydrate Active enZyme (CAZy) genes classified in different families and subfamilies numbers.

Specific FN	Genes per subclasses
AA3_2	36
CBM48	15
CBM50	24
CE14	263
CE5	9
CE9	29
GH1	132
GH114	1
GH13_11	15
GH13_29	128
GH13_30	39
GH13_31	235
GH135	2
GH15	48
GH2	46
GH23	152
GH24	18
GH25	103
GH29	2
GH3	3
GH32	165
GH33	11
GH36	13
GH43_30	4
GH65	27
GH68	6
GH76	132
GT1	78
GT11	36
GT2	1408
GT20	18
GT25	140
GT27	11
GT28	133
GT35	15

Supplementary Table 4. Total number of Carbohydrate Active enZyme (CAZy) genes classified in different families and subfamilies numbers.

Specific FN	Genes per subclasses
GT39	131
GT4	956
GT5	15
GT51	132
GT83	24
GT87	275
Total	**5043**

Supplementary Table 5. Nitrate-Nitrite metabolism genes sorted by isolation sources of *Rothia* species. Nitrate and nitrite metabolism Mb binding protein + Molybdopterins

Species	Strain ID	Isolation source	Nitrate and nitrite metabolism													Mb binding protein + Molybdopterins						
			aniA	*COG0778*	*hmp*	*narG*	*narK2*	*narK2-1*	*narX1*	*narX2*	*narY*	*nirB/nasD*	*nirD/nasE*	*norB*	*yodC*	*moaB*	*mobA*	*modA*	*moeA*	*moeB*	*mog*	*mopII*
Rothia amarae		*environment*	1	0	0	1	1	0	1	1	1	1	0	0	0	0	1	1	0	0	0	0
Rothia dentocariosa		*environment*	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1
Rothia dentocariosa		*environment*	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1
Rothia halotolerans		*environment*	0	0	0	1	1	1	1	1	1	0	0	0	0	0	1	0	0	1	0	0
Rothia kristinae		*environment*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0
Rothia kristinae		*environment*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0
Rothia kristinae		*environment*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0
Rothia kristinae		*environment*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0
Rothia kristinae		*environment*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0
Rothia kristinae		*environment*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0
Rothia kristinae		*environment*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0
Rothia kristinae		*environment*	0	0	0	1	1	1	1	1	1	0	0	0	0	0	1	0	0	1	0	0
Rothia kristinae		*environment*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
Rothia kristinae		*environment*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0
Rothia mucilaginosa		*environment*	1	0	0	1	1	0	1	1	1	1	0	0	0	0	1	1	0	0	0	0
Rothia sp.	*ND6WE1A*	*environment*	1	0	0	1	1	0	1	1	1	1	0	0	0	0	1	1	0	0	0	0
Rothia terrae		*environment*	1	0	0	1	1	0	1	1	1	1	1	0	0	0	1	0	0	0	0	0

Supplementary Table 5. Continued.

Species	Strain ID	Isolation source	Nitrate and nitrite metabolism													Mb binding protein + Molybdopterins						
			aniA	*COG0778*	*hmp*	*narG*	*narK2*	*narK2-1*	*narX1*	*narX2*	*narY*	*nirB/nasD*	*nirD/nasE*	*norB*	*yodC*	*moaB*	*mobA*	*modA*	*moeA*	*moeB*	*mog*	*mopII*
Rothia terrae		*environment*	1	0	0	1	1	0	1	1	1	1	1	0	0	0	1	0	0	0	0	0
Rothia dentocariosa		*food*	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1
Rothia dentocariosa		*food*	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1
Rothia koreensis		*food*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	0	0
Rothia koreensis		*food*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	0	0
Rothia sp.	*Olga*	*food*	1	1	1	1	2	2	1	1	1	1	1	1	0	1	1	1	1	0	1	1
Rothia kristinae		*human*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0
Rothia aeria	*F0474*	*human*	1	1	1	1	2	2	1	1	1	1	1	1	0	1	1	1	1	0	1	1
Rothia aeria	*F0184*	*human*	1	1	1	1	2	2	1	1	1	1	1	1	0	1	1	1	1	0	1	1
Rothia aeria		*human*	1	1	1	1	2	2	1	1	1	1	1	1	0	1	1	1	1	0	1	1
Rothia aeria		*human*	1	1	1	1	2	2	1	1	1	1	1	1	0	1	1	1	1	0	1	1
Rothia dentocariosa	*M567*	*human*	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1
Rothia dentocariosa	*ATCC17931*	*human*	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1
Rothia dentocariosa		*human*	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1
Rothia dentocariosa		*human*	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1
Rothia dentocariosa		*human*	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1
Rothia dentocariosa		*human*	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1
Rothia dentocariosa		*human*	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1
Rothia dentocariosa		*human*	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1
Rothia dentocariosa		*human*	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	2
Rothia dentocariosa		*human*	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1
Rothia mucilaginosa	*ATCC25296*	*human*	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	1

Rothia mucilaginosa	*M508*	*human*	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	1
Rothia mucilaginosa		*human*	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	1
Rothia mucilaginosa		*human*	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	1
Rothia mucilaginosa		*human*	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	1
Rothia mucilaginosa		*human*	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	1
Rothia mucilaginosa		*human*	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	1
Rothia mucilaginosa		*human*	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	2
Rothia mucilaginosa		*human*	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	1
Rothia mucilaginosa		*human*	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	1
Rothia mucilaginosa		*human*	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	1
Rothia mucilaginosa		*human*	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	1
Rothia mucilaginosa		*human*	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	1
Rothia mucilaginosa		*human*	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	1
Rothia mucilaginosa		*human*	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1
Rothia mucilaginosa		*human*	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	1
Rothia mucilaginosa		*human*	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	1
Rothia mucilaginosa		*human*	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	1
Rothia mucilaginosa		*human*	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	1
Rothia mucilaginosa		*human*	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	1
Rothia mucilaginosa		*human*	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	1
Rothia sp.	*HMSC08A08*	*human*	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1
Rothia sp.	*HMSC071B01*	*human*	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	1
Rothia sp.	*HMSC078H08*	*human*	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	1
Rothia sp.	*HMSC071F11*	*human*	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1
Rothia sp.	*HMSC058E10*	*human*	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1
Rothia sp.	*HMSC064D08*	*human*	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1
Rothia sp.	*HMSC072B03*	*human*	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	1
Rothia sp.	*HMSC069D01*	*human*	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	1
Rothia sp.	*HMSC075F09*	*human*	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	1

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Supplementary Table 5. Continued.

Species	Strain ID	Isolation source	Nitrate and nitrite metabolism													Mb binding protein + Molybdopterins						
			aniA	*COG0778*	*hmp*	*narG*	*narK2*	*narK2-1*	*narX1*	*narX2*	*narY*	*nirB/nasD*	*nirD/nasE*	*norB*	*yodC*	*moaB*	*mobA*	*modA*	*moeA*	*moeB*	*mog*	*moplI*
Rothia sp.	*HMSC067H10*	*human*	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1
Rothia sp.	*HMSC062H08*	*human*	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	1
Rothia sp.	*HMSC069C03*	*human*	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	1
Rothia sp.	*HMSC065G12*	*human*	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1
Rothia sp.	*HMSC065C12*	*human*	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	1
Rothia sp.	*HMSC069C10*	*human*	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	1
Rothia sp.	*HMSC065B04*	*human*	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	1
Rothia sp.	*HMSC068F09*	*human*	1	1	0	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	1
Rothia sp.	*HMSC069C04*	*human*	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	1
Rothia sp.	*HMSC073B08*	*human*	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	1
Rothia sp.	*HMSC066G02*	*human*	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	1
Rothia sp.	*HMSC068E02*	*human*	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	1
Rothia sp.	*HMSC061E04*	*human*	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	1
Rothia sp.	*HMSC072B04*	*human*	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	1
Rothia sp.	*HMSC071C12*	*human*	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	1
Rothia sp.	*HMSC072E10*	*human*	1	1	0	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	1
Rothia sp.	*HMSC036D11*	*human*	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1
Rothia sp.	*HMSC066G07*	*human*	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	1
Rothia sp.	*HMSC076D04*	*human*	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	1
Rothia sp.	*HMSC069C01*	*human*	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1
Rothia sp.	*HMSC066H02*	*human*	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	1
Rothia sp.	*HMSC065D09*	*human*	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	1

Rothia sp.	*HMSC065D02*	*human*	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1
Rothia sp.	*HMSC061C12*	*human*	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	1
Rothia sp.	*HMSC061D12*	*human*	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	1
Rothia sp.	*HMSC062F03*	*human*	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	1
Rothia sp.	*HMSC064F07*	*human*	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	1
Rothia sp.	*HMSC065C03*	*human*	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	1
Rothia sp.	*HSID18069*	*human*	1	1	1	1	2	2	1	1	1	1	1	1	0	1	1	1	1	0	1	1
Rothia sp.	*HSID18067*	*human*	1	1	1	1	2	2	1	1	1	1	1	1	0	1	1	1	1	0	1	1
Rothia aeria		*NA*	1	1	1	1	2	2	1	1	1	1	1	1	0	1	1	1	1	0	1	1
Rothia aeria		*NA*	1	1	1	1	2	2	1	1	1	1	1	1	0	1	1	1	1	0	1	1
Rothia aerolata		*NA*	1	0	0	1	1	0	1	1	1	1	1	1	0	0	1	1	0	0	0	0
Rothia kristinae	*NBRC15354*	*NA*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0
Rothia kristinae		*NA*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0
Rothia kristinae		*NA*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
Rothia mucilaginosa	*DY-18*	*NA*	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	1
Rothia aerolata	*159RC1*	*Porcine*	1	0	0	1	1	0	1	1	1	1	1	1	0	0	1	1	0	0	0	0
Rothia endophytica	*56QC2O2*	*Porcine*	1	0	0	1	0	0	1	1	1	1	1	0	0	0	1	1	0	0	0	0
Rothia nasimurium	*15QC4O2*	*Porcine*	1	0	0	1	0	0	1	1	1	1	1	0	0	0	1	0	0	0	0	0
Rothia nasimurium	*18QC4O2*	*Porcine*	0	1	0	1	0	0	1	1	1	1	1	0	0	0	1	0	0	0	0	0
Rothia nasimurium	*63RC1*	*Porcine*	1	0	0	1	0	0	1	1	1	1	1	0	0	0	1	0	0	0	0	0
Rothia nasimurium	*65.2RC1*	*Porcine*	1	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
Rothia nasimurium	*66RC1*	*Porcine*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
Rothia nasimurium	*67RC1*	*Porcine*	1	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
Rothia nasimurium	*123RC1*	*Porcine*	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
Rothia nasimurium	*124RC1*	*Porcine*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
Rothia nasimurium	*206RC1*	*Porcine*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
Rothia nasimurium	*213RC1*	*Porcine*	1	0	0	1	0	0	1	1	1	1	1	0	0	0	1	0	0	0	0	0
Rothia nasimurium	*2QC2O2*	*Porcine*	1	0	0	1	0	0	1	1	1	1	1	0	0	0	1	1	0	0	0	0
Rothia nasimurium	*19QC2O2*	*Porcine*	1	0	0	1	0	0	1	1	1	1	1	0	0	0	1	1	0	0	0	0

Supplementary Table 5. Continued.

Species	Strain ID	Isolation source	Nitrate and nitrite metabolism													Mb binding protein + Molybdopterins						
			aniA	*COG0778*	*hmp*	*narG*	*narK2*	*narK2-1*	*narX1*	*narX2*	*narY*	*nirB/nasD*	*nirD/nasE*	*norB*	*yodC*	*moaB*	*mobA*	*modA*	*moeA*	*moeB*	*mog*	*moplI*
Rothia nasimurium	*28QC2CO*	*Porcine*	1	0	0	1	0	0	1	1	1	1	1	0	0	0	1	1	0	0	0	0
Rothia nasimurium	*3QC2O2*	*Porcine*	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
Rothia nasimurium	*4QC2O2*	*Porcine*	1	0	0	1	0	0	1	1	1	1	1	0	0	0	1	1	0	0	0	0
Rothia nasimurium	*6QC2O2*	*Porcine*	1	0	0	1	0	0	1	1	1	1	1	0	0	0	1	1	0	0	0	0
Rothia nasimurium	*7QC2O2*	*Porcine*	1	0	0	1	0	0	1	1	1	1	1	0	0	0	1	0	0	0	0	0
Rothia nasimurium	*8QC2CO*	*Porcine*	1	0	0	1	0	0	1	1	1	1	1	0	0	0	1	1	0	0	0	0
Rothia nasimurium	*15QC2CO*	*Porcine*	1	0	0	1	0	0	1	1	1	1	1	0	0	0	1	0	0	0	0	0
Rothia nasimurium	*22QC2CO*	*Porcine*	1	0	0	1	0	0	1	1	1	1	1	0	0	0	1	0	0	0	0	0
Rothia nasimurium	*34QC2O2*	*Porcine*	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
Rothia nasimurium	*36QC2O2*	*Porcine*	1	0	0	1	0	0	1	1	1	1	1	0	0	0	1	1	0	0	0	0
Rothia nasimurium	*37QC2O2*	*Porcine*	1	0	0	1	0	0	1	1	1	1	1	0	0	0	1	1	0	0	0	0
Rothia nasimurium	*38QC2CO*	*Porcine*	1	0	0	1	0	0	1	1	1	1	1	0	0	0	1	1	0	0	0	0
Rothia nasimurium	*46QC2O2*	*Porcine*	1	0	0	1	0	0	1	1	1	1	1	0	0	0	1	0	0	0	0	0
Rothia nasimurium	*48QC2CO*	*Porcine*	1	0	0	1	0	0	1	1	1	1	1	0	0	0	1	0	0	0	0	0
Rothia nasimurium	*54QC2CO*	*Porcine*	1	0	0	1	0	0	1	1	1	1	1	0	0	0	1	0	0	0	0	0
Rothia nasimurium	*63QC2CO*	*Porcine*	1	0	0	1	0	0	1	1	1	1	1	0	0	0	1	0	0	0	0	0
Rothia nasimurium	*64QC2CO*	*Porcine*	1	0	0	1	0	0	1	1	1	1	1	0	0	0	1	0	0	0	0	0
Rothia nasimurium	*65RC1*	*Porcine*	1	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
Rothia nasimurium	*67.3RC1*	*Porcine*	1	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
Rothia nasimurium	*68RC1*	*Porcine*	1	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
Rothia nasimurium	*110RC1*	*Porcine*	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
Rothia nasimurium	*136RC1*	*Porcine*	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0

Rothia nasimurium	*207RC1*	*Porcine*	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
Rothia nasisuis	*107RC1*	*Porcine*	1	0	0	1	0	0	1	1	1	0	0	0	0	0	1	0	0	0	0	0
Rothia nasisuis	*31RC1*	*Porcine*	1	0	0	1	0	0	1	1	1	0	0	0	0	0	1	0	0	0	0	0
Rothia nasisuis	*69RC1*	*Porcine*	1	0	0	1	0	0	1	1	1	0	0	0	0	0	1	0	0	0	0	0
Rothia nasisuis	*141RC1*	*Porcine*	1	0	0	1	0	0	1	1	1	0	0	0	0	0	1	0	0	0	0	0
Rothia nasisuis	*152RC1*	*Porcine*	1	0	0	1	0	0	1	1	1	0	0	0	0	0	1	0	0	0	0	0
Rothia nasisuis	*196RC1*	*Porcine*	1	0	0	1	0	0	1	1	1	0	0	0	0	0	1	0	0	0	0	0
Rothia nasisuis	*32*	*Porcine*	1	0	0	1	0	0	1	1	1	0	0	0	0	0	1	0	0	0	0	0
Rothia kristinae		*animal*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0
Rothia nasimurium		*animal*	1	0	0	1	0	0	1	1	1	0	0	0	0	0	1	0	0	0	0	0
Rothia nasimurium		*animal*	1	0	0	1	0	0	1	1	1	1	1	0	0	0	1	0	0	0	0	0
Rothia nasimurium		*animal*	1	0	0	1	0	0	1	1	1	0	0	0	0	0	1	0	0	0	0	0
Rothia sp.	*ZJ1223*	*animal*	1	0	0	1	0	0	1	1	1	1	1	0	0	0	1	0	0	0	0	0
Rothia sp.	*ZJ932*	*animal*	1	0	0	1	0	0	1	1	1	1	1	0	0	0	1	0	0	0	0	0


bell hooks
Enedina Alves
Luiza Bairros
Simone Maia Evaristo
Marielle Franco
Angela Davis
#little Great things
SOU ZEZÉ SOU LEGI
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V

Microbiota-based interventions to provide colonisation resistance against *Streptococcus suis* in early life

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To be submitted

Abstract

Background: Microbiota of the tonsil and nasal epithelium is known to provide colonization resistance against pathogens and pathobionts that colonise the same niches. Porcine palatine tonsils are considered the major portal of entry for pathogenic serotypes of *Streptococcus suis* and are colonized by an extensive microbiota that is likely providing colonisation resistance against pathogenic *S. suis* and lower risk of invasive disease and transmission. Therefore, probiotic interventions based on core commensal species that are prevalent and abundant in healthy pigs are of great interest as a strategy to prevent dysbiosis of the microbiota and reduce risk of infectious diseases in farmed pigs.

Methods: We screened prevalent and abundant isolates of the tonsil microbiota for their potential to provide colonisation resistance against *S. suis* for example, by direct antagonism against of *S. suis* growth. We then tested the ability of different groups of orally administered probiotics to colonise the tonsil of piglets post-partum, modulate the microbiota and protect against *S. suis* challenge.

Results and conclusion: Differences in survival, lesion scores in susceptible piglets and changes in microbiota composition were observed between the probiotic and control groups after challenge with virulent *S. suis*. However due to an enteric *E.coli* infection in some of the piglets between day 2 and 5 the study lacked statistical power to draw firm conclusions. Strain-specific primers for two of the probiotics enabled us to show they colonised the tonsil. Not all piglets in each group developed clinical symptoms of *S. suis* but those that did, had higher relative abundances of *Alloprevotella*, *Porphyromonas* and *Moraxella* amplicon sequence variants (ASVs) in the tonsillar microbiota; the corresponding nasal microbiota composition was not significantly different.

Keywords: *Streptococcus suis*, microbiota, probiotics, upper respiratory tract

Introduction

Body sites of eukaryote hosts are colonized by a diverse microbial community that is likely to influence occurrence of infectious diseases, by interfering with invasion of exogenous pathogens and/or expansion of local pathobionts, a process referred to as colonisation resistance. In pigs, colonisation resistance plays a role in suppressing pathogenic members *Streptococcus, Actinobacillus, Pasteurella,* and *Bordetella,* all common colonizers of the palatine tonsils (1). In young piglets *Streptococcus suis* disease is a serious welfare problem and significant cause of mortality around the time of weaning. Cases of sepsis and meningitis due to *S. suis* are one of the main reasons to prescribe antibiotics on pig farms.

Antibiotic use is linked to emergence and spread of antibiotic resistance but due to the lack of cross-protective vaccines (2) or other preventative strategies they are routinely used to treat *S. suis* disease and spread of infections on affected farms. *S. suis* infections of pigs and humans are associated with various diseases including pneumonia, endocarditis, septicaemia, and meningitis (3). Based on capsular polysaccharide (CPS) composition, *S. suis* has been classified into 35 different serotypes (1–34 and 1/2) of which 29 are still being recognized (2, 4). *S. suis* also poses a public health threat due to high zoonotic potential of specific serotypes with a global distribution (5). Capsular serotype 2 is the most prevalent serotype associated with pig- and human infections. Infections due to serotype 9 have recently increased in Europe, Canada and China (6). Genomic studies on *S. suis* isolates collected from systemic body sites of diseased pigs and the tonsil surface of piglets on farms without disease has shown that the *S. suis* population consists of non-disease associated strains with a commensal association with the host as well as highly pathogenic isolates with smaller genomes and genes linked to pathogenesis (7).

To cause invasive disease *S. suis* must cross host barriers, evade the host immune system, spread and proliferate in the bloodstream and organs (8) including the brain. The dynamics of clinical disease, the mechanisms by which *S. suis* can invade the body and spread via the blood, and the pathobiology have not been well characterised for pig and human hosts. *S. suis* is a common colonizer of the nasal cavity and the tonsils of piglets which are considered to be the main portal of entry facilitating invasive disease (5). However, the intestine and respiratory airway are also potential portals of entry for pathogenic isolates of *S. suis* (9, 10).

The microbiota communities colonising host mucosal sites play an important role in protection against pathogen colonization and overgrowth of indigenous pathobionts (11). Infection by pathogens can correlate with major changes in the microbiota

composition, or dysbiosis, with loss of diversity and, consequently, loss of microbial protection and colonization resistance. *S. su*is case-control studies on the tonsil microbiota has shown significant differences in bacterial composition between healthy and *S. suis* diseased animals (12, 13), which may have consequences for host health. Furthermore, in pre-weaning piglets a lower diversity of the tonsil microbiota is significantly correlated with occurrence of invasive disease post-weaning (12).

The main mechanisms of colonisation resistance are nutrient depletion by microbiota growth, microbiota-induced stimulation of innate and adaptive immune responses and direct inhibition of pathogen growth by microbiota-derived substances (11). For instance, probiotic mechanisms include direct antagonism against pathogens or indirect beneficial effects on host immunity and microbiota homeostasis (14). Many probiotic species are able to neutralize toxins, reduce infection and incidences of antibiotic-associated diarrhoea (15) whereas some (for example, *Enterococcus faecalis* strains) can inhibit *Clostridium difficile* while maintain the structural integrity of the liver and intestinal cells (16).

Introducing microbial species that engage in mutualistic or competitive interactions with co-occurring microbes may promote development of a homeostatic microbiota that provides colonisation resistance and is resilient to perturbations. Probiotic interventions are of great interest to improve animal performance and reduce the use of antibiotics to treat and prevent infectious diseases in farmed pigs (17). We hypothesized that certain prevalent species of core tonsil commensal genera might antagonize *S. suis* and promote development of synergetic microbiota of tonsil or nasal cavity, and that such taxa may reduce invasive disease caused by virulent *S. suis*. To test the selected microbial consortia, we inoculated piglets in early life with different combinations of four bacterial isolates and challenged *S. suis* serotype 2 strain under controlled conditions.

Material and Methods

1. Selection of candidate probiotics

The candidate probiotic strains were initially selected based on prevalence and abundance in healthy piglets from a cross-sectional microbiota study on European farms in the Netherlands, Germany, and Spain (12, 18, 19). Selected taxa were then obtained from our large culture collection of tonsil-associated isolates (Chapter III). Other selection criteria were: (i) adherence to 2D monolayers of porcine tonsil organoid cells, (ii) sensitivity to antibiotics (iii) sensitivity to active porcine serum (iv)

activity against *S. suis* (Supplementary Table 1). To investigate adherence to 2D tonsil organoids, cells were seeded in a 96 well flat bottom plate and grown as a monolayer in LWRN medium until they reached 90–100% confluence (approx. 1–2x10E+4 per well). Bacteria from overnight cultures were pelleted by centrifugation, resuspended in Hank's Balanced Salt Solution (HBSS) and added to each well at a ratio of 10 bacteria per cell and incubated for 1 hr at 37ºC. Tonsil cells were washed with PBS, detached from the plate and re-suspended in HBSS to estimate the proportion of bacteria bound per tonsil cell by flow cytometry (Cytoflex and CytExpert software, Beckman Coulter Inc., Brea, CA, USA).

Bacterial isolates were tested for sensitivity to eight antibiotics used treat infections in pigs: chloramphenicol (C), ampicillin (AMP), polymyxin (PB), doxilin (DO), penicillin G (P), erythromycin (E), ciprofloxacin (CIP), gentamicin (CN) using Neo-Sensitabs (Rosco Diagnostica) and measuring the diameter of bacterial growth inhibition after overnight incubation at 37 °C. Diameters of inhibition zones were compared with the manufacturer's breakpoint tables. Sensitivity to serum was tested by incubation of bacteria in active porcine serum (Gibco, Fisher Scientific). A bacterial suspension of each strain was prepared in PBS with 20% glycerol to reach an OD600 of 0.3 in a SpectraMax M5 (Molecular Devices) (20). Then 100 µl of bacterial suspension (approx. 10E+6 colony forming units [CFU]/mL) were mixed with 900 µl of fresh filtered porcine serum in three 1.5 ml Eppendorf vials per bacterial strain and incubated for 3 h at 37 °C prior to plating on BHI or TSY agar plates. Bacterial survival was calculated by comparing bacterial CFU counts at time 0 and 3 h after incubation in porcine serum.

The *S. suis* inhibition screening assays were performed using over-night (o/n) liquid cultures generated from pure bacterial colonies previously described (Chapter III). To assess inter-probiotic competition, for each strain, pure cultures inoculated in the centre of solid agar media in Petri dishes were spread with a sterile loop down the centre to create 3–5 mm wide lawns and allowed to grow until they reach confluence. The other assayed isolates, taken from established colonies in separate Petri dishes, were inoculated in triplicate within 1–2 mm from the lawns by cross-streaking; the perpendicular inoculations were made using sterile small loops per Petri dish; replicates were inoculated onto different plate (21). Growth of test strains was considered inhibited if a clearing zone $\geq$ 5 mm was observed in the area adjacent to the lawn in at least two of three replicate assays.

2. Bacterial cultures

Selected probiotic candidates were cultured aerobically in BHI (Brain Heart Infusion Broth, Himedia, Mumbai, India) medium at 37 °C supplemented with 5% CO_2 for 18h-24h. *S. suis* strains were cultured in THY (Todd-Hewitt Broth enriched with 0.2% yeast extract, Oxoid, Basingstoke/UK).

3. Bacterial DNA extraction and sequencing

Genomes of the *Streptococcus suis* strain 12RC1, *Rothia nasimurium* 63RC1 and *Rothia nasisuis* 31RC1, *Moraxella pluranimalium* LG6-2 strains used in this study were sequenced using Illumina HiSeq 2000 (Illumina Inc., San Diego, CA, USA) and assembled using SPAdes version 3.7 (22) as described previously and in Chapter III and IV. Additional genomes from other strains were downloaded from RefSeq genome database of the National Center for Biotechnology Information (NCBI) for genomic analyses and primer design (see section below).

4. Strain specific primer design and qPCR

To analyse the prevalence of the inoculated commensals in the tonsil microbiota, four pairs of strain-specific quantitative PCR primers and probes were designed. First, whole genome sequences of the strains selected to be included in probiotic cocktails were analysed *in silico* by (i) identifying the strain-specific sequences; (ii) validating the putative strain-specific regions using BLAST; (iii) designing specific primer/probe sets for qPCR, and (iv) validating the specificity of the qPCR sets *in vitro* using closely phylogenetically related strains (23, 24). Strain-specific genomic regions were selected based on gene presence-absence as inferred from orthologous groups constructed with orthaGogue (25). *In silico* validations were first performed using a local BLAST database that included over 80 whole genome sequences from each species and also closely related species (Supplementary Table 2); the strain-specific sequence fragments were used as queries for a BLASTn similarity search with default parameters. In a second validation step, strain-specific fragments were subjected to a BLASTn search against the nonredundant NCBI databases, and four sequence fragments with no match in the BLAST sequence analyses were kept as putative strain-specific sequence for the designing of specific primer/probe sets for qPCR. To assay the prevalence of *S. suis* in tonsil microbiota samples, two pairs of strain-specific quantitative PCR primers and probes previously designed and validated were used in our analyses. Primer and probe specificity were evaluated *in silico* using full 16S rRNA gene alignments to other *Streptococcus* species. Virulence marker 1 (VM1) is a highly conserved gene that is present in genomes of disease-associated strains (i.e., present in 95%-99% of all strains within highly pathogenic clades) and is absent in all clades containing mostly non-disease-associated *S. suis* strains (unpublished data HMI and UCAM).

Taqman qPCR primers were designed using Beacon Designer v.8.21 from Premier Biosoft International, Palo Alto, CA, using parameters compatible with qPCR probe detection (Supplementary Table 3). The qPCR detection of each strain utilised forward and reverse primers and a TaqMan probe annealing to regions within ORFs annotated as hypothetical proteins. Primer/probe specificity was checked using gDNA isolated from 4-5 strains from each species and an unrelated DNA fragment of a similar size as negative control (Supplementary Table 4). Standard curves were generated with known concentrations of the products from the purified PCR fragments containing upstream sequences region of each strain-specific fragment under study, serially diluted from 10E+8 to 10E+1 copies of each target gene per mL. Analysis of the qPCR data was performed using Rotor-gene Q® software (Qiagen). Statistical analyses were performed using GraphPad Prism Ver. 9.2.0 for Windows (GraphPad, San Diego,USA). We tested for statistical differences using analysis of variance (ANOVA) and the nonparametric Kruskal-Wallis test.

5. *In vivo* experiment and sampling strategy

Two weeks before the expected farrowing date, four sixth-parity sows were obtained from farms that had reported no or low incidence of *S. suis* disease and absence of *S. suis* serovar 2 based on PCR detection in DNA from the vaginal cavity of sows and nasal cavity of piglets, as previously described (26). To reduce the effects of maternal microbiota transfer during birth, the sows were treated with a systemic antimicrobial (ceftiofur) and vaginal lavage (phenoxymethylpenicillin) prior to farrowing. Piglets, weaned early from their sows approximately 12 hours after birth, were randomized and put in one out of four treatment groups or the control group.

Candidate probiotic cocktails consisting of 4 isolates were orally (1 mL) and nasally (0.5mL per nostril) administered to the piglets (Table 1). Prior to their introduction, bacterial numbers were estimated and adjusted to a concentration of approximately 10E+5CFU/mL for day 1 and on day 6 to 10E+7 CFU/mL of each isolate. The control group of piglets was orally (1 mL) and nasally (0.5mL per nostril) inoculated with 1X phosphate-buffered saline (PBS) solution (pH ~ 7.4). The controlled colonization experiment consisted of one control group (C1 not administered oral probiotics) and four groups orally and nasally inoculated with different combinations of candidate probiotics (G2-G5) (Table 2). On day 31, all piglets were challenged by nasal (0.5 mL per nostril) inoculation with 10E+10CFU/mL of the virulent *S. suis* strain P1/7 (Figure 1). Due to an *Escherichia coli* infection causing severe diarrhoea, all piglets had to be treated with colistin on days 3,4 and 5 after birth, and 19 piglets with clinical symptoms were removed from the study between day 4 and 6 and euthanized to prevent suffering.

Palatine tonsil of the piglets were sampled using HydraFlock® Swabs (Puritan, USA) on days 14, 28 and at necropsy time points (between day 32 and the end of the experiment) (Figure 1) for DNA isolation. The swabs were cut at the breakpoint (20 mm) and inserted directly into PowerBead Tubes (MoBio, Qiagen) prefilled with 750 µL of Power Bead lysis solution and garnet beads (0.7 mm Ø size). After sampling, PowerBead Tubes were stored at -80°C until processing. After day 31, piglets were observed daily. Fever, apathy, dyspnea, prostration, stiffness, lameness, tremors, or convulsions were expected after experimental infection with virulent *S. suis*. Animals showing signs of infection and suffering before the end of the experiment, were considered symptomatic (S) and euthanized by an intravenous sodium pentobarbital overdose (200 mg/kg, Dolethal, Vetoquinol SA). Surviving piglets, considered non-symptomatic (NS), were euthanized at the end of the study on day 41 (Figure 1). Mortality associated to *S. suis* infection was confirmed by post-mortem examination and bacterial isolation from the lesions.

6. Microbiota analyses

The 16S rRNA gene V3-V4 region from tonsillar samples were amplified with primers 341F (CCTAYGGGRBGCASCAG) and 806R (GGACTACNNGGGTATCTAAT) and from nasal samples with the primers F (CCTACGGGNGGCWGCAG) and R (NACTACHVGGGTATCTAATCC); all V3-V4 regions were sequenced by Novaseq 6000 (Novogene Co., Ltd.). Reads were trimmed with cutadapt 2.3 (27) using default settings before being processed in DADA2 (28) following the v1.4 workflow for paired end data. Taxonomy was assigned with SILVA database v138 (29). Amplicon sequence variants (ASVs) with taxonomic assignment as eukaryote or chloroplast were discarded, and read counts were rarefied to the minimum library size for tonsil (31192 reads) and nasal (44635 reads). Alpha and beta diversity were calculated using R packages Phyloseq (30) and vegan (31), and the adonis function in vegan was used to perform multivariate PERMANOVA test for the statistical significance between groups. Multiple testing correction was calculated by false discovery rate (FDR). In the cross-sectional study bacterial taxa with a minimum abundance of 0.1% in > 90% of all samples from healthy piglets were considered core members of porcine tonsil microbiota.

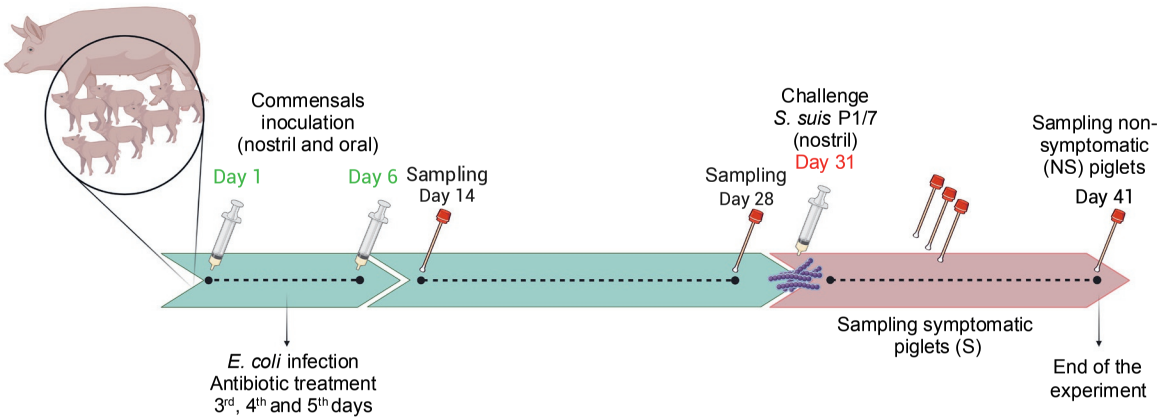

Figure 1. Schematic representation of the *in vivo* study displaying timepoints for the nasal and oral inoculation, challenge with virulent *S. suis*, and sampling. Day 1 and Day 6 mark the two inoculation days with the four isolates used in this study: *R. nasimurium, R. nasisuis, S. suis and M. pluranimalium*. Day 31 marks the inoculation of *S. suis* virulent strain. Green arrow represents the period prior the inoculation of the *S. suis* virulent strain. Pink arrow represent the period after challenge with the virulent *S. suis* P1/7. Not symptomatic (NS): piglets without any symptoms or only mild symptons that survived the experiment; Symptomatic (S): piglets which developed major symptoms and were euthanised before the end of the experiment to avoid suffering.

Results

Selection of candidate probiotics

A total of 53 isolates were selected for evaluation as candidate probiotics following a range of criteria (see Methods section 1 and Supplementary Table 1). These isolates corresponded to prevalent and abundant ASVs in the tonsil microbiota of healthy piglets and inhibited *S. suis* growth *in vitro* (see Methods and Chapter III). The 53 candidate isolates included 15 isolates of the genus *Rothia*, 26 isolates of the genus *Moraxella* and 12 putatively non-pathogenic and commensal isolates of *S. suis*. The non-pathogenic *S. suis* isolates were selected based on their full genome sequence, phylogenetic clustering in non-disease-associated clades, lack of genes conserved in virulent strains and presence of putative natural antibiotic-encoding genes. Following testing as outlined in the methods section, nine isolates were selected for antibiotic resistance assays with eight antibiotics used to treat bacterial infections in pigs (Supplementary Table 1). We selected 4 isolates for *in vivo* colonization: 1 *Rothia nasisuis* (31RC1), 1 *Rothia nasimurium* (63RC1), 1 *S. suis* (12RC1), and 1 *Moraxella pluranimalium* (LG62), based on characteristics listed in Table 1 and in the text below. In these final selected four isolates we also assessed their activity in active porcine serum resistance assays and direct challenge (inter-microbe competition) assays.

The genome of *Rothia nasisuis* isolate 31RC1 encodes a non-ribosomal peptides (NRPS) biosynthetic gene cluster producing valinomycin, a natural antibiotic with activity against *S. suis in vitro* (33). This isolate adhered to tonsil epithelial organoids (11%) *in vitro*, and was sensitive to seven of the eight antibiotics tested (Table 1). Although prevalent in piglet tonsil microbiota, this taxon is found at relatively low abundance (0.006% at ASV level; Chapter III). The second isolate, *Rothia nasimurium* 63RC1 had 0.5% relative abundance in tonsil microbiota. *R. nasimurium* 63RC1 did not inhibit *S. suis in vitro* but corresponded to an ASV that was negatively correlated with *S. suis* disease in cross-sectional studies of the tonsil microbiota (Chapter III). *Moraxella* strain LG62 was among the most abundant ASVs in the tonsil associated microbiota with relative abundance of 5.3%. Moreover, *M. pluranimalium* LG62 can form biofilms and adhere to A549 alveolar epithelial cell lines and tonsil epithelial organoids (10%) and was sensitive to porcine serum. LG62 was also sensitive to different antibiotics (34). Three of the four selected probiotics candidates (31RC1, 63RC1 and LG62) carry in the genome biosynthetic gene clusters (BGCs) predicted to produce betalactones, biologically relevant molecules that include diverse, different natural bioactive compounds that occur as precursors in different bacterial biochemical pathways (35). Lastly, *S. suis* strain 12RC1 serotype 31 was among the more abundant ASV in the tonsil microbiota with (2.1% relative abundance at ASV

Table 1. Overview of the characteristics of the 4 selected candidate probiotics.

ID	Species	Adherence assays (%)	Inhibiting growth of *S. suis*	Correlation with *S. suis*	C	Antibiotics (diameter (mm))							Serum Resistance Assay	antiSMASH
						AMP	PB	DO	P	E	CIP	CN		
12RC1	*S. suis*	13%	-	NA	S (20)	R (17)	R (0)	R (10)	R (12)	S (22)	S (18)	S (15)	R	bacteriocin/ RaS-RiPP
31RC1	*R. nasisuis*	11%	+	NA	S (30)	S (33)	S (12)	S (30)	S (30)	R (0)	S (19)	S (18)	R	NRPS/ betalactone
63RC1	*R. nasimurium*	1%	-	NEG	S (30)	R (16)	R (10)	S (21)	R (15)	S (30)	R (9)	S (16)	R	betalactone
LG6-2	*M. pluranimalium*	10%	-	NEG	S (34)	S (20)	S (20)	S (25)	R (15)	S (26)	S (36)	S (20)	S	betalactone

(12RC1) commensal *Streptococcus suis* strain; (31RC1) *Rothia nasisuis* strain; (63RC1) *Rothia nasimurium* strain; (LG6-2) *Moraxella pluranimalium* strain. (R) Resistant; (S) Sensitive; (NEG) Negatively correlated (Chapter III)), NA: not applicable; antiSMASH: software to identify, annotate, and compare gene clusters that encode the biosynthesis of secondary metabolites (32); NRPS: non-ribosomal peptides; RiPP: post-translationally modified peptides; adherence (%): ratio of adhered bacteria per tonsil cell.

level). The selected probiotic candidates did not inhibit each other, enabling them to be combined as the different consortia. To assess performance of these 4 consortia as potential probiotics, we performed an *in vivo* colonisation and *S. suis* challenge study in piglets.

Piglet colonization and challenge with *S. suis* serotype 2

Candidate probiotic cocktails containing commensal strains (see section above) were inoculated on day 1 and day 6 according to the four-groups design (G2-G5) with different combinations of strains (Table 2); animals in control group (C1) were not inoculated with commensals. The experiment had been designed with an initial number of 44 healthy piglets. Despite administering the antibiotic colistin on days 3,4 and 5, 19 piglets with severe diarrhoea due to an *E. coli* infection were euthanised to avoid further suffering. The experiment was continued with 25 animals, (Figure 1).

On day 31, all 25 piglets from the five groups (C1, G2-G5) were inoculated with virulent *S. suis* strain P1/7 (serotype 2). The disease severity was scored before euthanasia between 0-5; no, mild and severe clinical signs. After day 31, 14 piglets had clinical symptoms of *S. suis* infection. Symptomatic piglets were euthanized while 11 piglets remained healthy with no or very mild symptoms over the 41 days (Figure 1).

Colonization of piglets with the candidate probiotic cocktails and protection against *S. suis*-associated disease

To assess presence and prevalence of the inoculated commensals (days 1 and 6) as well as total *S. suis* abundance in the tonsil and nasal microbiota, specific quantitative PCR (qPCR) was performed with samples collected on day 14 and day 28 before challenge with *S. suis* and one at necropsy time points when the experiment ended (Figure 1). The two qPCR primer-probe sets designed to specifically quantify isolates 12RC1 and 31RC1 (see Methods section 4) generated an amplicon in samples from uninoculated piglets. This indicated that strains present in the tonsil microbiota possess the same DNA sequence but their genome sequences were not available in open access databases used by us to validate the strain-specific qPCR. This prevented us from quantifying the target copy numbers of *R. nasisuis* 31RC1 and *S. suis* 12RC1 in tonsil swab DNA.

In contrast, the probiotic candidates *R. nasimurium* 63RC1 and *M. pluranimalium* LG62 were only detected in the piglets inoculated with these strains, enabling us to quantify colonisation over time. After (8 days) colonisation the abundance of these two probiotics was 10E+1 - 10E+3 target copies in tonsils and nasal swabs (Supplementary Figure 1). *S. suis* represent about 5 to 10% relative abundance of microbiota are detected at 10E+05 - 10E+07 target copies per swab. However, it is

Table 2. Overview of the treatments per group including numbers of piglets that survived the challenge and numbers of piglets that were euthanized due to clinical signs.

Group	Probiotic Treatment	Animal ID	Day of the Necropsy	Total no. of piglets on day 6#	Total no. of Symptomatic piglets (S)	Total no. of Non Symptomatic piglets (NS)
C1	Control	1	34	5	3	2
		12	41			
		17	41			
		22	36			
		27	37			
G2	12RC1	8	41	2	0	2
		14	41			
G3	63RC1 + LG6-2 + 12RC1	9	35	6	5	1
		15	41			
		20	36			
		25	37			
		33	34			
		37	34			
G4	31RC1 + 63RC1 + LG6-2 + 12RC1	5	36	6	4	2
		10	37			
		16	41			
		21	35			
		26	41			
		38	35			
G5	31RC1 + 63RC1 + LG6-2	2	34	6	2	4
		7	37			
		13	41			
		18	41			
		23	41			
		28	41			

Commensal *Streptococcus suis* strain 12RC1; *Rothia nasisuis* strain 31RC1; *Rothia nasimurium* strain 63RC1 and *Moraxella pluranimalium strain* LG6-2. Symptomatic (S): piglets euthanized due to clinical symptoms of *S. suis* disease; Non- Symptomatic (NS) No or only mild symptoms of *S. suis* disease after challenge. # indicates number of piglets in each group after removal of animals with diarrhoea on day 5.

important to consider that there are approximately 4 copies of the 16S RNA gene in the genome of *S. suis* which means we detected around 2.50E+04 - 2.50E+06 genome equivalents 2uL of DNA isolated from swab.

Overall, inoculation with the candidate probiotic cocktails did not lead to significant differences in microbiota composition or in total *S. suis* abundance between uninoculated control or other groups, although differences in survival and changes in microbiota composition were observed between the probiotic and control groups after challenge with virulent *S. suis* (Figure 2 and Supplementary Figure 2).

To visually link the time points to the respective changes in relative abundance of certain taxa, principal component analysis (PCA) of all samples based on microbiota sequencing partial 16S rRNA gene sequences was carried out. PCA showed separated clusters of composition at the first time point (day 14) versus composition at day 28 and necropsy time points. The microbiota composition at day 14 was different to that on day 28 and necropsy time points which clustered together in the PCA (Figure 3). These trends were the same for tonsil and nasal microbiota composition. However, the nasal microbiota had lower species richness compared to tonsillar samples.

Susceptibility to the challenge with virulent *S. suis*

Due to an *E. coli* infection, 19 of the 44 piglets had to be removed from the study on days 2-6, as mentioned earlier. This left too few piglets for meaningful statistical comparison of the effect of the probiotics on susceptibility to *S. suis* infection. The remaining piglets which were symptomatic or non-symptomatic to the *S. suis* challenge on day 31 is summarized in Table 2. When analyzing the survival of the piglets after the challenge with P1/7, we observed some differences between the groups (Table 2). 40% of the piglets in the control non-inoculated piglets (C1 group) survived the challenge with virulent *S. suis*. Survival was lower in the G4 (33.3%) and G3 (16.6%,) inoculated piglets. However, the survival was highest in G5 inoculated with the 31RC1, 63RC1 and LG62, with 66.6% of the piglets surviving until the end of the experiment. The two piglets in G2 remained healthy during the challenge and survived until the end of the study.

The clinical signs after challenge were compatible with *S. suis* disease and at necropsy time points the lesions were also characteristic of *S. suis* infection, including serofibrinous pericarditis, peritonitis and pleuritis, and fibrinopurulent meningitis. The group G5 showed less severity of lesions as measured by a lesion score (Table 3), coinciding with the best survival (Table 2). The cause of the lesions and disease was confirmed by isolation of challenge strain P1/7 from samples taken from the affected organs.

A

Day 14					Day 28					Necropsy						
C1	G2	G3	G4	G5	**C1**	G2	G3	G4	G5	**C1**	G2	G3	G4	G5	Mean	ASVs	
31.1	29.9	35.9	40.1	35.7	64.7	36.8	46.9	51.2	53.3	38.4	39.9	30.6	43.1	50.5	41.9	36	*Actinobacillus*
48.6	53.6	44.0	45.5	45.7	13.2	32.7	24.2	13.7	13.9	12.4	27.6	11.0	10.7	15.0	27.5	29	*Streptococcus*
6.6	0.8	4.9	2.2	1.0	6.9	4.7	5.5	10.9	11.4	16.4	4.0	16.1	12.6	11.9	7.7	10	*Moraxella*
0.2	0.3	1.3	0.8	0.1	0.8	5.5	4.6	4.6	2.0	0.6	11.5	1.5	3.1	0.5	2.5	23	*Prevotella*
6.1	4.9	2.1	1.9	9.0	0.8	2.8	1.6	0.9	1.0	0.6	1.1	0.4	1.2	0.6	2.3	14	*Lactobacillus*
0.1	0.1	0.2	0.1	0.1	0.1	0.2	0.7	0.5	0.3	3.6	1.1	12.6	7.7	6.3	2.2	8	*Alloprevotella*
0.1	0.6	1.0	0.3	0.2	0.3	0.2	0.8	0.8	0.5	6.1	0.8	9.3	6.6	4.8	2.2	11	*Porphyromonas*
2.0	0.5	0.8	0.6	0.4	3.6	2.9	3.0	3.1	4.4	2.6	2.5	1.4	1.4	2.0	2.1	5	*Rothia*
0.4	0.2	0.3	0.4	0.5	0.8	0.3	1.0	0.8	1.0	1.7	0.5	1.8	1.4	1.0	0.8	7	*Acinetobacter*
0.3	0.2	0.8	0.6	0.2	0.7	0.3	0.7	0.8	0.6	1.1	0.4	1.4	0.8	0.7	0.6	2	*Neisseria*
0.1	0.5	0.4	0.1	0.2	0.0	0.0	0.2	0.1	0.1	2.9	0.2	0.2	0.2	0.8	0.4	4	*Bacteroides*
0.1	0.4	0.3	0.3	0.1	0.1	0.2	0.3	0.1	0.2	0.4	0.1	1.1	1.3	0.8	0.4	2	*Veillonella*
0.1	0.1	0.2	0.1	0.0	0.4	1.2	0.7	0.9	0.6	0.4	0.6	0.1	0.3	0.2	0.4	7	*Agathobacter*
0.3	0.2	0.6	0.5	0.6	0.2	1.0	0.7	0.2	0.2	0.0	0.2	0.1	0.1	0.1	0.3	1	*Escherichia−Shigella*
0.0	0.0	0.1	0.0	0.0	0.3	0.6	0.6	0.5	0.6	0.6	0.7	0.3	0.2	0.1	0.3	10	UCG−005
0.5	1.4	0.8	0.7	0.8	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.3	4	*Staphylococcus*
0.0	0.0	0.1	0.0	0.0	0.2	0.5	0.4	0.3	0.4	0.3	0.4	0.1	0.2	0.1	0.2	2	*Clostridium sensu stricto* 1
0.0	0.0	0.1	0.0	0.0	0.6	0.3	0.4	0.5	0.5	0.2	0.3	0.0	0.1	0.0	0.2	7	*Roseburia*
0.0	0.0	0.1	0.0	0.0	0.2	0.2	0.4	0.6	0.3	0.3	0.3	0.1	0.1	0.1	0.2	5	*Christensenellaceae* R−7 group
0.0	0.0	0.1	0.1	0.0	0.1	0.1	0.1	0.2	1.0	0.5	0.3	0.1	0.1	0.1	0.2	6	*Prevotellaceae* NK3B31 group

B

Day 14					Day 28					Necropsy						
C1	G2	G3	G4	G5	**C1**	G2	G3	G4	G5	**C1**	G2	G3	G4	G5	Mean	ASVs	
22.2	33.6	0.2	31.6	0.2	32.5	30.8	7.4	19.8	15.1	22.5	36.3	11.6	26.8	40.0	22.0	8	*Moraxella*
10.9	2.2	13.8	10.5	4.3	8.5	24.9	27.3	25.4	8.4	4.7	19.0	18.4	21.2	2.2	13.4	66	*Prevotella*
13.1	24.2	13.3	15.5	16.4	6.2	3.9	2.6	3.3	9.0	5.5	5.1	3.5	4.1	8.9	9.0	15	*Streptococcus*
4.5	2.5	6.1	3.5	4.7	1.5	1.9	1.8	2.8	3.6	2.0	1.7	2.1	4.0	1.9	3.0	18	*Lactobacillus*
6.2	12.1	2.1	0.0	8.3	2.6	1.4	0.9	0.8	0.1	1.7	0.0	0.4	2.6	0.2	2.6	4	*Bergeyella*
1.7	0.3	4.3	2.0	2.0	2.3	0.6	1.4	1.5	4.9	6.5	1.3	2.0	1.2	2.8	2.3	36	*Prevotellaceae* NK3B31 group
1.7	0.6	2.6	1.6	2.9	1.7	1.8	3.5	2.5	2.5	3.3	1.8	3.2	1.4	2.6	2.2	26	*Rikenellaceae* RC9 gut group
0.4	0.3	0.6	0.4	0.7	2.1	1.8	3.8	2.3	2.3	3.9	1.7	3.8	1.5	2.2	1.9	36	UCG−005
0.9	0.4	1.3	0.9	1.4	2.3	1.4	2.3	1.5	2.4	3.8	1.9	3.0	1.8	2.4	1.9	13	*Clostridium sensu stricto* 1
0.4	0.4	1.9	0.9	1.2	1.7	2.7	3.0	3.1	1.9	1.3	1.2	2.0	1.7	1.0	1.6	18	*Agathobacter*
1.4	0.2	1.8	1.0	1.7	0.5	1.4	1.9	1.6	1.1	0.9	1.8	2.9	3.0	0.6	1.5	22	*Alloprevotella*
0.3	0.2	0.5	0.4	0.8	1.1	1.0	4.2	3.1	1.1	2.8	0.7	2.4	0.6	1.9	1.4	22	*Christensenellaceae* R−7 group
0.5	1.4	1.8	1.3	1.5	2.9	0.1	0.1	1.0	0.3	5.1	0.1	0.6	1.3	1.8	1.3	2	*Rothia*
0.5	0.2	0.7	0.4	0.2	2.1	0.8	2.4	1.6	2.1	1.6	0.5	1.6	0.8	1.8	1.2	24	*Roseburia*
1.1	0.2	1.7	0.9	1.7	0.9	1.2	0.8	1.1	1.2	1.1	1.3	1.2	1.9	0.9	1.1	22	*Blautia*
0.6	0.4	1.5	0.9	0.9	1.8	0.7	1.3	0.9	1.7	2.6	0.4	1.4	0.6	1.5	1.1	27	*Ruminococcus*
2.8	1.3	5.7	1.9	3.0	0.0	0.1	0.6	0.3	0.1	0.0	0.1	0.5	0.1	0.1	1.1	21	*Bacteroides*
1.4	0.4	1.7	1.0	2.0	1.7	0.2	0.3	0.4	2.4	1.1	0.9	0.8	0.6	1.2	1.1	12	*Treponema*
0.7	0.6	1.3	1.0	0.5	0.6	1.3	0.7	0.8	1.0	0.7	1.4	1.1	1.2	0.3	0.9	7	*Subdoligranulum*
1.2	0.2	1.9	0.7	1.8	0.4	0.9	0.5	0.7	0.7	0.2	0.4	0.5	1.3	0.2	0.8	10	*Faecalibacterium*

Figure 2. Heatmap displaying relative abundance values for the most abundant genera at day 14, day 28 and necropsy time points of the tonsillar **(A)** and nasal **(B)** microbiota from groups (C1, G2-G5).

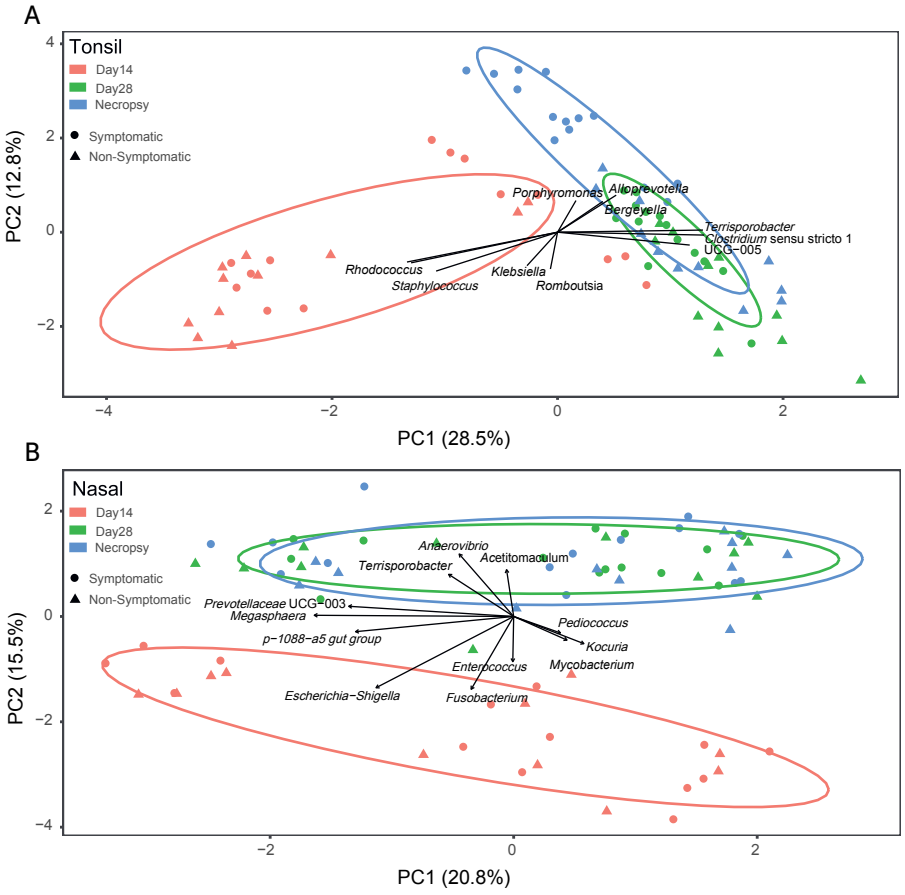

Figure 3. PCA of all samples from the three time points. A) In the tonsil samples the two axes (PC1 and PC2) explain 28.5% and 12.8% of the total variation. B) In the nasal samples the axes explain 20.8% and 15.5% of the total variation, respectively. The dots represent single samples and when close together correspond to observations that have similar scores on the PCA components. Pink represents all samples from Day 14; green are the samples from Day 28; blue are the samples from necropsy time points. Each ellipse shows a 75% confidence for each group. The samples in the direction of the arrows have higher abundance of the genus indicated. The length of an arrow indicates the relative importance of the genus. Smaller angles between the arrow and axis represent a higher correlation between the two values. (S) Symptomatic; (NS) Non-Symptomatic (section below).

Table 3. Mean lesion score at necropsy per animal and group.

Groups	Mean lesion score
C1	4.2
G2	NA
G3	3.8
G4	4.8
G5	2.7

The lesion scores were assessed ranging from a scale of 0 (no lesion) to 5 (most severe lesion). Higher value of the mean lesion score is as of result greater number of lesions and/or with more severity in piglets from the respective inoculated. NA; non applicable.

To correlate piglet mortality and survival to *S. suis* abundance, we used qPCR to quantify of total *S. suis* and virulent *S. suis* in piglets across the three timepoints (see Methods section 4). The *S. suis* abundance in tonsillar and nasal microbiota was detected during the first days of life, amounting to between 10E+3 and 10E+6 16S rRNA gene target copies per sample (Figure 4). No significant differences were detected in the amounts of total *S. suis* between groups in tonsil and nasal samples (Supplementary figure 2), although abundance of total *S. suis* was significantly higher in the tonsil than in the nasal samples. In the tonsillar samples, total *S. suis* abundance significantly increased between the first timepoint (day 14) and the two later timepoints (day 28 to day 41) ($p < 0.001$) (Figure 4A) but no differences were found between sampling days for the nasal samples (Figure 4B).

S. suis strains qPCR-positive for the VM1 gene were only detected after challenge with *S. suis* strain P1/7 on day 31, except for one tonsil sample from one piglet (ID23) at day 28 in group G5. Symptomatic piglets which developed severe symptoms of *S. suis* disease before the end of the experiment carried greater number of pathogenic *S. suis* (VM1 target copies) in the tonsil than the non-symptomatic piglets ($p < 0.01$) (Figure 5A). In addition, in the nasal cavity pathogenic *S. suis* was only detected in the symptomatic piglets (Figure 5B).

Differences in microbiota of symptomatic or non-symptomatic piglets

The tonsil microbiota of symptomatic and non-symptomatic piglets showed high relative abundance of members from the genera *Actinobacillus, Streptococcus* and *Moraxella* as previously reported for microbiota of healthy piglets (Chapter III, (12)). We did not find significant differences in the composition of the tonsil microbiota between S and NS piglets, but we did find significant differences in relative abundance of certain genera between the sampling days. At day 14, there was a higher relative abundance of *Streptococcus* compared to the other two timepoints.

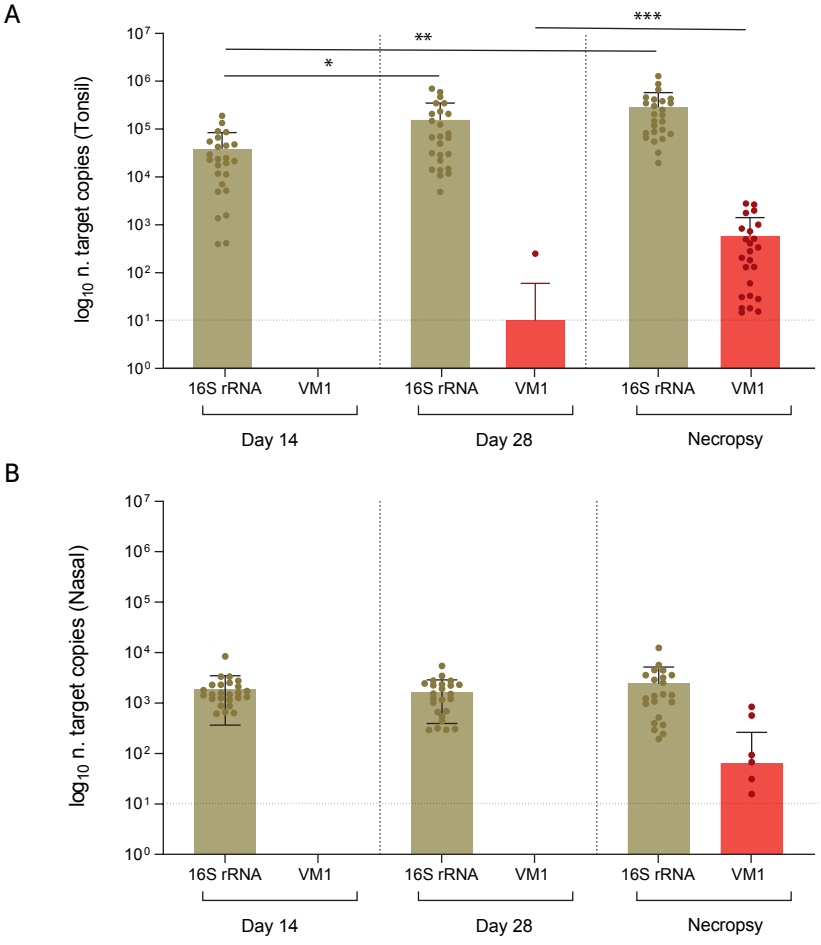

Figure 4. Quantification of total amount of *S. suis* and VM1-positive *S. suis* in tonsil **(A)** and nasal samples **(B)**. Quantification of the total amount of *S. suis* based on target copies of 16S rRNA gene (khaki) and of the virulent strains based on target copies of virulence marker VM1 (red) on days of sampling. Error bars show standard deviation. Horizontal dashed lines denote threshold of detection (10E+1 target copies). (*) indicates statistically significant differences in the target copies of the 16S RNA gene of *S. suis* in tonsillar samples.

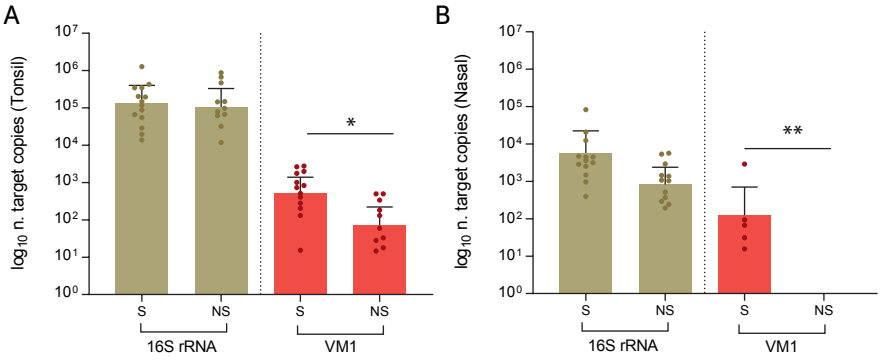

Figure 5. qPCR quantification of *S. suis* 16S rRNA target gene copies and virulence marker (VM1) copies in tonsillar **(A)** and nasal **(B)** samples from piglets pooled based on susceptibility to infection after challenge with virulent *S. suis* on day 31. A) Quantification of *S. suis* (16S rRNA and VM1 qPCR target copies) present in the tonsil samples of piglets that survived the duration of the experiment to day 41); non-symptomatic (NS) and symptomatic (S) indicates piglets euthanized due to severity of clinical signs between day 31 and 41 ; B) Quantification of *S. suis* (16 rRNA and VM1 qPCR target copies) present in the nasal samples of piglets. Khaki bars: total amount of *S. suis* (16S rRNA gene); red bars: target copy number of virulent strain (VM1 marker). Dots represent single samples. Error bars show standard deviation. (*) indicates statistically significant differences in the amount of the 16S RNA gene or VM1 gene of *S. suis* in tonsillar samples.

The tonsil microbiota composition of piglets that had developed *S. suis* infection showed significantly higher relative abundance (ASVs level) of members of *Alloprevotella*, *Porphyromonas* and *Moraxella* genera (FDR < 0.001), compared to non-symptomatic piglets whereas the NS piglets had significantly higher relative abundance of *Actinobacillus* and *Lactobacillus* (FDR < 0.00) (Figure 6A). In contrast the nasal microbiota composition did not show significant changes in microbiota of symptomatic piglets at necropsy which may be due to *S. suis* invasion of the tonsils and localized inflammation (Figure 6B).

Based on the Shannon diversity calculations per time point, day 14 tonsil samples had significantly lower alpha diversity compared to day 28 and at necropsy time points (Figure 7). At necropsy, the microbiota showed a trend of increased alpha diversity, without statistically significant differences compared to alpha diversities calculated for day 14 and day 28 (Figure 7A). In the tonsil samples, no significant differences in diversity were observed between symptomatic and non-symptomatic piglets. In the nasal samples, symptomatic (S) piglets had higher but not significant Shannon diversity than the NS piglets at necropsy time points (Figure 7B).

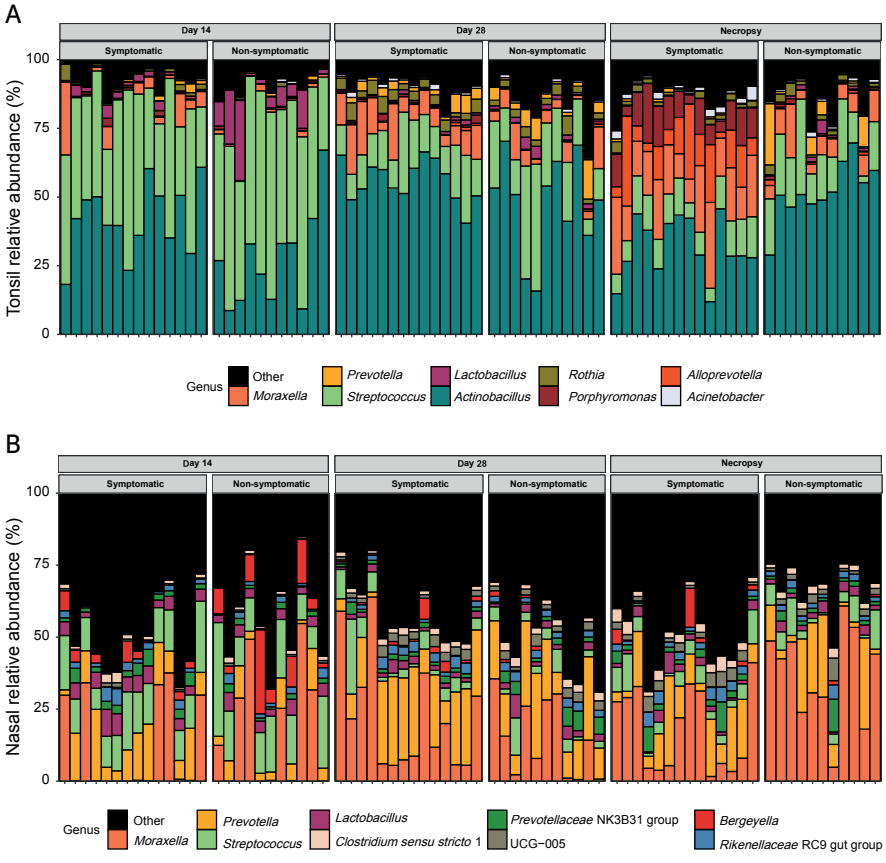

Figure 6. Microbiota composition at three timepoints of symptomatic and non-symptomatic piglets after challenge by virulent *S. suis* challenge strain P1/7. **(A)** Tonsillar microbiota samples; **(B)** nasal microbiota samples.

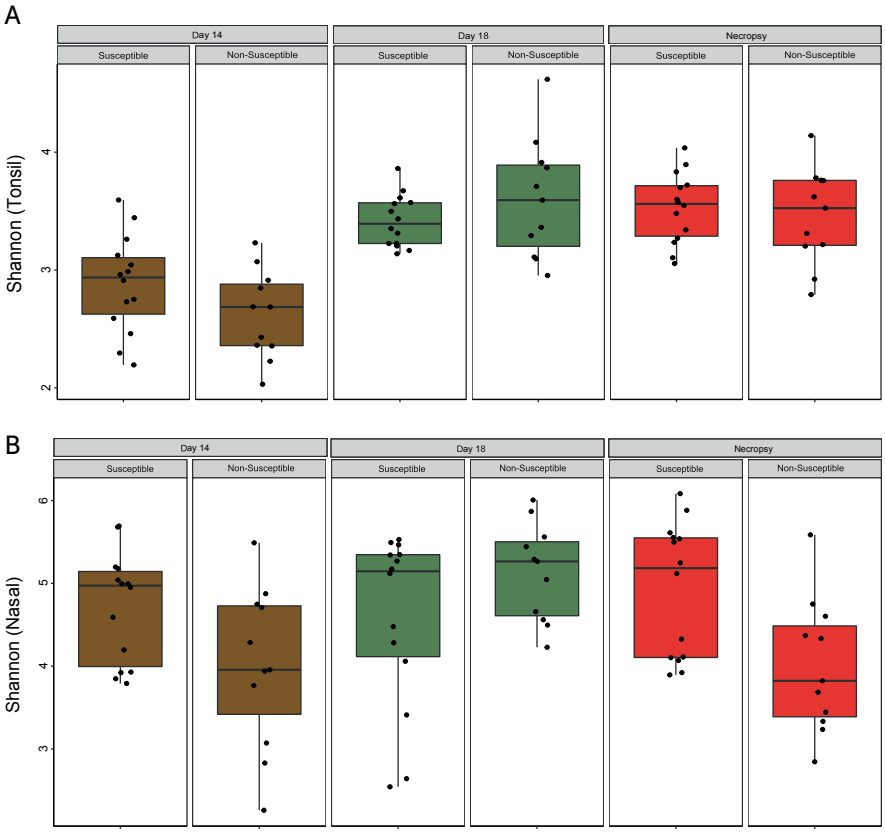

Figure 7. Alpha diversity (Shannon Index of diversity based on amplicon sequencing data) of tonsillar (A) and nasal (B) samples per time point grouped in symptomatic (S) and non-symptomatic (NS) samples. The line inside the box represents the median, while the whiskers represent the lowest and highest values within the 1.5 interquartile range (IQR). Plots per time points: Symptomatic (red/pink) and non-symptomatic (dark green/light green) Each dot represents data from individual piglet swabs.

Discussion

Several animal and human infectious diseases are associated with altered microbiota composition at mucosal sites, which can impact on disease pathology and reduce colonization resistance to pathogens (36, 37). The composition of the microbiota is strongly influenced by maternal transmission and the environmental exposure (38). The vertical transmission can greatly affect colonization resistance to pathogens. Here we isolated and characterized prevalent health -associated bacteria from the tonsil microbiota with potential to colonize the tonsil and inhibit growth of *S. suis* a major pathogen of piglets around the period of weaning.

One of the candidate probiotics, 31RCI, had *in vitro* activity against *S. suis* and two (63RC1 and LG6-2) were significantly negatively correlated with *S. suis* in a previous case control study across different farms and European countries (12, 39). All candidate probiotic genomes harbored BGCs which are implicated in production of anti-microbials which might not have been expressed *in vitro*. We also selected a non-pathogenic isolate of *S. suis* as a potential competitor of pathogenic strains based on its genome phylogeny, and lack of markers and capsule serotypes found in pathogenic strains. Non-pathogenic strains of *S. suis* have larger genomes than pathogenic strains containing accessory genes likely involved in their commensal lifestyle (40). The notion that pathogenic *S. suis* strains compete with commensal *S. suis* for host colonization is also supported by the finding that pathogenic strains are 100 to 1000-fold lower in abundance on the tonsil as shown in our own results from qPCR.

Cocktails of the candidate probiotics strains were administered to piglets at day 1 and day 6 after birth to assess their ability to colonize, impact on microbiota development and protection against challenge with a pathogenic strain of *S. suis*. Although, we developed strain-specific qPCR assays based on sequenced genomes in databases and our own collection of isolates, only two were suitable to monitor colonization. Thus we could establish that *R. nasimurium* 63RC1 and *M. pluranimalium* LG6-2 colonized tonsil and nasal cavity of the piglets after inoculation. In the colonization model contact with the sow was limited to the maximum of 12 hours and then piglets were artificially fed. This period of contact with the sow was expected to allow maternal transfer of microbiota which also occurs from exposure to the vaginal microbiota during birth (41). The reduced colostrum intake may have led to the *E. coli* infection from day 2 to 5 which meant that piglets had to be treated with colistin on days 3, 4 and 5 and the removal of 19 piglets from the study due to their symptoms. Another factor which is known to cause post-weaning diarrhea and increase the risk of ETEC infection is separation from the sow which is normally at

the point of weaning (42). This stressor is thought to be due to stress hormone effects on intestinal permeability and barrier function (43). Despite the antibiotic administration until day 5, two of the administered probiotics were shown to colonize the tonsil but we cannot exclude the possible influence of colistin on the microbiota and on the outcomes of the study (43).

The microbiota of the remaining piglets that had not suffered from *E. coli* infection was monitored on day 14, 28 and at necropsy time points. Different mortality rates were observed between the groups which received different candidate probiotic cocktails and the control groups. Furthermore encouraging results were observed in the group 5 (G5), inoculated with *R. nasisuis* 31RC1, *R. nasimurium* 63RC1 and *M. pluranimalium* LG6-2, which had the highest number of non-symptomatic pigs and lowest lesion scores in the pigs that were euthanised due to symptoms. We did not measure significant differences in the composition of the microbiota nor in the amount of *S. suis* detected by qPCR between the groups. The abundance and diversity of the airway microbiome can favor or not the colonization of virulent strains of *S. suis*, and consequentially influence the host's health. The qPCR results showed that amounts of commensal and virulent strains of *S. suis* were significantly higher in the tonsillar samples compared to nasal samples. We also detected more virulent strains in the tonsil of the piglets with clinical signs of *S. suis* infection while in the nose of same piglets none or very low amounts of virulent strains were observed. This result is in accordance with the notion that palatine tonsils are the main niche of *S. suis* and that the main route of invasion is via these tonsils (44). The age of the piglets may also have played a role in the establishment of the species in the tonsil, as the total amount of *S. suis* was higher at the last two time points.

Not all piglets developed severe infections after the challenge with virulent *S. suis*, suggesting that other factors influence development and progression of *S. suis* infection. We hypothesized that differential taxon abundance in the nasal and tonsil microbiota might be one relevant factor. In tonsil microbiota we observed an increase of the relative abundance of disease-associated taxa such as *Alloprevotella* and *Porphyromonas* (12). Disease-associated taxa might have positive interactions with *S. suis,* for instance by providing metabolites and/or antagonizing competitors of *S. suis*. On the other hand, specific *Moraxella* and *Streptococcus* ASVs had higher abundance in the tonsillar and nasal microbiota of the NS piglets which could indicate beneficial roles for these ASVs, for instance in the stability and restoration of the microbiota.

The piglets were negative for the VM1 virulence marker for pathogenic strains before the challenge with *S. suis* P1/7. After the challenge, all piglets were positive for VM1 in which 14 were symptomatic piglets and 11 were non-symptomatic piglets. Analysis of the microbiota composition in piglets which developed clinical signs of *S. suis* infection did not show significant higher species level abundance of *S. suis*, which was confirmed by qPCR for total *S. suis*. Microbiota samples from the tonsil and nose of infected piglets with clinical signs of *S. suis* disease had significantly more qPCR target copies representing the *S. suis* virulence marker VM1 than healthy piglets.

Streptococcus is one of the most abundant genera in the porcine upper respiratory tract and also part of the tonsillar core microbiota (18, 38, 41). At the first sampling time point, day 14, we observed a high amount of *S. suis*. *S. suis* is highly diverse species with about 29 recognized serotypes, however, pig infections are usually caused by virulent strains from a few specific serotypes (45), emphasizing that virulence is not a common characteristic of the species *S. suis*. The observation that clinical signs of *S. suis* disease appeared only after inoculation with the virulent serotype 2 strain of *S. suis* also emphasizes that most of *S. suis* colonizing piglets in early life are commensal members of the species that contribute to establishment of healthy microbiota in the upper respiratory tract and might be competitors of pathogenic strains (46).

Conclusion

The application of candidate probiotics did not sufficiently protect piglets against challenge by a virulent *S. suis* strain, with variable mortality rate at the endpoint one month after challenge. The inoculated commensal probiotic strains did not colonise to high relative abundance and did not appear to lead to a significantly different microbiota composition or different diversity compared to the control group under the given experimental conditions. We did measure clear changes in tonsillar microbiota composition following the inoculation of the virulent *S. suis* strain. Some of the taxa that increased in abundance after inoculation with virulent *S. suis* may have symbiotic interactions with virulent *S. suis* strains, and this notion is of sufficient interest to investigate in future studies.

Ethical statement

The use of experimental animals in the current study was based on an assessment of animal welfare. The research protocol was designed in accordance with the ethical standards approved by the Ethical Commission in Animal Experimentation

of the Generalitat de Catalunya (protocol approval no. 10201) upon formal application and in accordance with the EU Directive 2010/63/EU and the Spanish regulations (Real Decreto 53/2013).

Data availability statement

The sequencing data are available at NCBI under the following BioProjects: PRJNA862074 for the whole-genome sequencing and PRJNA928212 for the microbiota sequencing data All data generated for this study are included in the manuscript and/or in the Supplementary Files.

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Supplementary Material

Supplementary Table 1. Overview of the characteristics of the 53 isolates for evaluation of probiotic traits, including the 4 selected candidate probiotics.

	LAB_ID	Genome size (bp)	GC (%)	Species	Serotype	BAPS	BAPS Proportion Clinical	Pathogenic Clade	Country/site	Adherence assays
Tonsil isolates	12RC1	2157327	48	*S. suis*	31	9	14%	NonPath	Spain_tonsil-swabs	13%
	120RC1	2158422	62	*S. suis*	31	9	14%	NonPath	Spain_tonsil-swabs	10%
	27RC1	2384308	98	*S. suis*	4	23	3%	NonPath	Spain_tonsil-swabs	25%
	28RC1	2385480	101	*S. suis*	4	23	3%	NonPath	Spain_tonsil-swabs	21%
	29RC1	2382005	103	*S. suis*	4	23	3%	NonPath	Spain_tonsil-swabs	30%
	198RC1	2386023	96	*S. suis*	4	23	3%	NonPath	Spain_tonsil-swabs	14%
	DNC15	2,22		*S. suis*		8	22%	NonPath	Denmark	7%
	DNC12	2,41		*S. suis*		9	14%	NonPath	Denmark	23%
	WB4_2_C14	2,42		*S. suis*		21	29%	NonPath	Spain	4%
	DNC39	2,56		*S. suis*		21	29%	NonPath	Denmark	2%
	DNC37	2,58		*S. suis*		21	29%	NonPath	Denmark	-
	DNC13	2,41		*S. suis*		16	27%	NonPath	Denmark	5%
Tonsil isolates	31RC1	2719140	57,99	*Rothia nasisuis*	NA	NA	NA	NA	Spain_tonsil-swabs	11%
	152RC1	2714093	57,95	*Rothia nasisuis*	NA	NA	NA	NA	Spain_tonsil-swabs	5%
	196RC1	2781870	57,8	*Rothia nasisuis*	NA	NA	NA	NA	Spain_tonsil-swabs	12%
	652RC1	2467431	59,95	*Rothia nasisuis*	NA	NA	NA	NA	Spain_tonsil-swabs	2%
	67RC1	2472103	59,96	*Rothia nasisuis*	NA	NA	NA	NA	Spain_tonsil-swabs	2%
	69RC1	2702694	57,99	*Rothia nasisuis*	NA	NA	NA	NA	Spain_tonsil-swabs	18%
	141RC1	2753241	57,88	*Rothia nasisuis*	NA	NA	NA	NA	Spain_tonsil-swabs	7%
	66RC1	2430530	60,51	*Rothia nasisuis*	NA	NA	NA	NA	Spain_tonsil-swabs	1%
	123RC1	2396918	60,23	*Rothia nasisuis*	NA	NA	NA	NA	Spain_tonsil-swabs	1%
	124RC1	2419319	60,04	*Rothia nasisuis*	NA	NA	NA	NA	Spain_tonsil-swabs	-
	206RC1	2381635	60,15	*Rothia nasisuis*	NA	NA	NA	NA	Spain_tonsil-swabs	-
	159RC1	2468432	58	*Rothia nasimurium*	NA	NA	NA	NA	Spain_tonsil-swabs	4%
	148RC1	2477919	57,91	*Rothia nasimurium*	NA	NA	NA	NA	Spain_tonsil-swabs	4%
	63RC1	2410350	59,66	*Rothia nasimurium*	NA	NA	NA	NA	Spain_tonsil-swabs	1%
	213RC1	2409647	59,66	*Rothia nasimurium*	NA	NA	NA	NA	Spain_tonsil-swabs	1%

			Antibiotics										
Activity against S. suis	Inter-probiotic competition	Correlation with S. suis	C	AMP	PB	DO	P	E	CIP	CN	Serum Resistance Assay	antiSMASH	Observations
-	-	NA	20	17	-	10	12	22	18	15	R	bacteriocin/RaS-RiPP	
-		NA										bacteriocin/RaS-RiPP	
-		NA										bacteriocin/lassopeptide	
-		NA										bacteriocin/lassopeptide	
-	-	NA	20	15	-	10	10	15	15	15		bacteriocin/lassopeptide	
-		NA										bacteriocin/lassopeptide	
-		NA										-	
-		NA										-	
+		NA										bacteriocin/T3PKS	
-		NA										-	
-		NA										-	
-		NA										-	
+	-		30	33	12	30	30	-	19	18	R	NRPS/betalactone	
+	-		30	33	15	33	36	-	16	10		NRPS/betalactone	
+	-		30	30	10	20	30	10	15	20		NRPS/betalactone	
-												NRPS/betalactone	
-												NRPS/betalactone	
-												NRPS/betalactone	
+	-		35	36	14	30	30	-	15	16		NRPS/RaS-RiPP	
-												NRPS	
-												NRPS	
-												NRPS	
-												NRPS	
-												betalactone	
-												betalactone	
-	-	NEG	30	16	10	21	15	30	9	16	R	betalactone	
-	-	NEG	32	15	-	20	12	32	10	17		betalactone	

Supplementary Table 1. Continued.

	LAB_ID	Genome size (bp)	GC (%)	Species	Serotype	BAPS	BAPS Proportion Clinical	Pathogenic Clade	Country/site	Adherence assays
Nasal isolates	AR5A	NA	NA	*Moraxella sp.*	NA	NA	NA	NA	Spain_nasal-swabs	-
	GM3-2	NA	NA	*Moraxella sp.*	NA	NA	NA	NA	Spain_nasal-swabs	5%
	EJ43-3A	NA	NA	*Moraxella sp.*	NA	NA	NA	NA	Spain_nasal-swabs	3%
	CR-10	NA	NA	*Moraxella sp.*	NA	NA	NA	NA	Spain_nasal-swabs	25%
	CR-18	NA	NA	*Moraxella sp.*	NA	NA	NA	NA	Spain_nasal-swabs	-
	GM8-1	NA	NA	*Moraxella sp.*	NA	NA	NA	NA	Spain_nasal-swabs	-
	GM5-1	NA	NA	*Moraxella sp.*	NA	NA	NA	NA	Spain_nasal-swabs	12%
	EJ44-2A	NA	NA	*Moraxella sp.*	NA	NA	NA	NA	Spain_nasal-swabs	20%
	EJ45-1			*Moraxella sp.*	NA	NA	NA	NA	Spain_nasal-swabs	-
	LG6-7g	NA	NA	*Moraxella sp.*	NA	NA	NA	NA	Spain_nasal-swabs	11%
	KD4-7	NA	NA	*Moraxella sp.*	NA	NA	NA	NA	Spain_nasal-swabs	13%
	LG6-2			*Moraxella sp.*	NA	NA	NA	NA	Spain_nasal-swabs	10%
	LG6-4	NA	NA	*Moraxella sp.*	NA	NA	NA	NA	Spain_nasal-swabs	-
	VL6-4	NA	NA	*Moraxella sp.*	NA	NA	NA	NA	Spain_nasal-swabs	16%
	LL-3	NA	NA	*Moraxella sp.*	NA	NA	NA	NA	Spain_nasal-swabs	-
	UK1-12	NA	NA	*Moraxella sp.*	NA	NA	NA	NA	Spain_nasal-swabs	16%
	UK1-20	NA	NA	*Moraxella sp.*	NA	NA	NA	NA	Spain_nasal-swabs	12%
	VL9-7	NA	NA	*Moraxella sp.*	NA	NA	NA	NA	Spain_nasal-swabs	16%
	GM5-7	NA	NA	*Moraxella sp.*	NA	NA	NA	NA	Spain_nasal-swabs	-
	GM5-5	NA	NA	*Moraxella sp.*	NA	NA	NA	NA	Spain_nasal-swabs	-
	CR-7A	NA	NA	*Moraxella sp.*	NA	NA	NA	NA	Spain_nasal-swabs	-
	VL3-9	NA	NA	*Moraxella sp.*	NA	NA	NA	NA	Spain_nasal-swabs	-
	VL1-4	NA	NA	*Moraxella sp.*	NA	NA	NA	NA	Spain_nasal-swabs	-
	VL6-6	NA	NA	*Moraxella sp.*	NA	NA	NA	NA	Spain_nasal-swabs	-
	VL1-5			*Moraxella sp.*	NA	NA	NA	NA	Spain_nasal-swabs	-
	VL2-5	NA	NA	*Moraxella sp.*	NA	NA	NA	NA	Spain_nasal-swabs	-

Activity against S. suis	Inter-probiotic competition	Correlation with S. suis	Antibiotics								Serum Resistance Assay	antiSMASH	Observations
			C	AMP	PB	DO	P	E	CIP	CN			
-		NEG										NA	only 16S rRNA gene
-		NEG										NA	only 16S rRNA gene
-		NEG										NA	only 16S rRNA gene
-		NEG										NA	only 16S rRNA gene
-		NEG										NA	only 16S rRNA gene
-		NEG										NA	only 16S rRNA gene
-		NEG										NA	only 16S rRNA gene
-		NEG										NA	only 16S rRNA gene
-		NEG										no BGCs	
-		NEG										NA	only 16S rRNA gene
-		NEG										NA	only 16S rRNA gene
-	-	NEG	34	20	20	25	15	26	36	20	R	betalacone	
-		NEG										NA	only 16S rRNA gene
-		NEG										NA	only 16S rRNA gene
-		NEG										NA	only 16S rRNA gene
-		NEG										NA	only 16S rRNA gene
-		NEG										NA	only 16S rRNA gene
-		NEG										NA	only 16S rRNA gene
-												NA	only 16S rRNA gene
-												NA	only 16S rRNA gene
-												NA	only 16S rRNA gene
-												NA	visible growth in BHI after 48 hours
-												NA	visible growth in BHI after 48 hours
-												NA	visible growth in BHI after 48 hours
-												betalacone/RiPP-like	
-												NA	only 16S rRNA gene

Supplementary Table 2. Strains used for full genome comparison.

Species	Strain	Accession number	Extra information		
Streptococcus suis	21402_NLC18	colab. UCAM	Non-Clinical	*Sus scrofa*	Netherlands
Streptococcus suis	21417_NLC33	colab. UCAM	Non-Clinical	*Sus scrofa*	Netherlands
Streptococcus suis	21397_NLC13	colab. UCAM	Non-Clinical	*Sus scrofa*	Netherlands
Streptococcus suis	21401_NLC17	colab. UCAM	Non-Clinical	*Sus scrofa*	Netherlands
Streptococcus suis	21400_NLC16	colab. UCAM	Non-Clinical	*Sus scrofa*	Netherlands
Streptococcus suis	21385_NLC1	colab. UCAM	Non-Clinical	*Sus scrofa*	Netherlands
Streptococcus suis	21420_NLC36	colab. UCAM	Non-Clinical	*Sus scrofa*	Netherlands
Streptococcus suis	21406_NLC22	colab. UCAM	Non-Clinical	*Sus scrofa*	Netherlands
Streptococcus suis	21298_NLR14	colab. UCAM	Respiratory	*Sus scrofa*	Netherlands
Streptococcus suis	21296_NLR12	colab. UCAM	Respiratory	*Sus scrofa*	Netherlands
Streptococcus suis	21329_NLR45	colab. UCAM	Respiratory	*Sus scrofa*	Netherlands
Streptococcus suis	21319_NLR35	colab. UCAM	Respiratory	*Sus scrofa*	Netherlands
Streptococcus suis	21310_NLR26	colab. UCAM	Respiratory	*Sus scrofa*	Netherlands
Streptococcus suis	21322_NLR38	colab. UCAM	Respiratory	*Sus scrofa*	Netherlands
Streptococcus suis	21318_NLR34	colab. UCAM	Respiratory	*Sus scrofa*	Netherlands
Streptococcus suis	21292_NLR8	colab. UCAM	Respiratory	*Sus scrofa*	Netherlands
Streptococcus suis	21333_NLR49	colab. UCAM	Respiratory	*Sus scrofa*	Netherlands
Streptococcus suis	21330_NLR46	colab. UCAM	Respiratory	*Sus scrofa*	Netherlands
Streptococcus suis	21320_NLR36	colab. UCAM	Respiratory	*Sus scrofa*	Netherlands
Streptococcus suis	21291_NLR7	colab. UCAM	Respiratory	*Sus scrofa*	Netherlands
Streptococcus suis	21290_NLR6	colab. UCAM	Respiratory	*Sus scrofa*	Netherlands
Streptococcus suis	21300_NLR16	colab. UCAM	Respiratory	*Sus scrofa*	Netherlands
Streptococcus suis	21361_NLS27	colab. UCAM	Sys-Brain	*Sus scrofa*	Netherlands
Streptococcus suis	21380_NLS46	colab. UCAM	Sys-Brain	*Sus scrofa*	Netherlands
Streptococcus suis	21368_NLS34	colab. UCAM	Sys-Brain	*Sus scrofa*	Netherlands
Streptococcus suis	21348_NLS14	colab. UCAM	Sys-Brain	*Sus scrofa*	Netherlands

Streptococcus suis	21353_NLS19	colab. UCAM	Sys-Brain	*Sus scrofa*	Netherlands
Streptococcus suis	21352_NLS18	colab. UCAM	Sys-Brain	*Sus scrofa*	Netherlands
Streptococcus suis	21347_NLS13	colab. UCAM	Sys-Brain	*Sus scrofa*	Netherlands
Streptococcus suis	21357_NLS23	colab. UCAM	Sys-Brain	*Sus scrofa*	Netherlands
Streptococcus suis	21350_NLS16	colab. UCAM	Sys-Brain	*Sus scrofa*	Netherlands
Streptococcus suis	21358_NLS24	colab. UCAM	Sys-Brain	*Sus scrofa*	Netherlands
Streptococcus suis	21369_NLS35	colab. UCAM	Sys-Brain	*Sus scrofa*	Netherlands
Streptococcus suis	19894_SS15144_T1_C16	colab. UCAM	Non-Clinical	*Sus scrofa*	Spain
Streptococcus suis	19879_M101999_C1	colab. UCAM	Non-Clinical	*Sus scrofa*	Spain
Streptococcus suis	19892_WB4_2_C14	colab. UCAM	Non-Clinical	*Sus scrofa*	Spain
Streptococcus suis	19886_GM8_2_C8	colab. UCAM	Non-Clinical	*Sus scrofa*	Spain
Streptococcus suis	19891_WB2_3_C13	colab. UCAM	Non-Clinical	*Sus scrofa*	Spain
Streptococcus suis	19885_GM2_2_C7	colab. UCAM	Non-Clinical	*Sus scrofa*	Spain
Streptococcus suis	19880_M104170_C2	colab. UCAM	Non-Clinical	*Sus scrofa*	Spain
Streptococcus suis	19889_VL5_4_C11	colab. UCAM	Non-Clinical	*Sus scrofa*	Spain
Streptococcus suis	19874_M107244_R46	colab. UCAM	Respiratory	*Sus scrofa*	Spain
Streptococcus suis	19834_M103246_R6	colab. UCAM	Respiratory	*Sus scrofa*	Spain
Streptococcus suis	19858_M105281_R30	colab. UCAM	Respiratory	*Sus scrofa*	Spain
Streptococcus suis	19848_M107687_R20	colab. UCAM	Respiratory	*Sus scrofa*	Spain
Streptococcus suis	19850_M104827_R22	colab. UCAM	Respiratory	*Sus scrofa*	Spain
Streptococcus suis	19853_M104928_R25	colab. UCAM	Respiratory	*Sus scrofa*	Spain
Streptococcus suis	19877_M108095_R49	colab. UCAM	Respiratory	*Sus scrofa*	Spain
Streptococcus suis	19843_M103975_R15	colab. UCAM	Respiratory	*Sus scrofa*	Spain
Streptococcus suis	19866_M106456_R38	colab. UCAM	Respiratory	*Sus scrofa*	Spain
Streptococcus suis	19836_M103466_R8	colab. UCAM	Respiratory	*Sus scrofa*	Spain
Streptococcus suis	19847_M104396_R19	colab. UCAM	Respiratory	*Sus scrofa*	Spain
Streptococcus suis	19841_M103904_R13	colab. UCAM	Respiratory	*Sus scrofa*	Spain
Streptococcus suis	19859_M105435_R31	colab. UCAM	Respiratory	*Sus scrofa*	Spain
Streptococcus suis	19860_M105455_R32	colab. UCAM	Respiratory	*Sus scrofa*	Spain

Supplementary Table 2. Continued.

Species	Strain	Accession number	Extra information		
Streptococcus suis	19826_M107147_S48	colab. UCAM	Sys-Brain	*Sus scrofa*	Spain
Streptococcus suis	19809_M105623_S31	colab. UCAM	Sys-Brain	*Sus scrofa*	Spain
Streptococcus suis	19790_M103642_S12	colab. UCAM	Sys-Brain	*Sus scrofa*	Spain
Streptococcus suis	19794_M103947_S16	colab. UCAM	Sys-Brain	*Sus scrofa*	Spain
Streptococcus suis	19806_M105244_S28	colab. UCAM	Sys-Brain	*Sus scrofa*	Spain
Streptococcus suis	19802_M105040_S24	colab. UCAM	Sys-Brain	*Sus scrofa*	Spain
Streptococcus suis	19803_M105040_S25	colab. UCAM	Sys-Brain	*Sus scrofa*	Spain
Streptococcus suis	19798_M104300_S20	colab. UCAM	Sys-Brain	*Sus scrofa*	Spain
Streptococcus suis	19785_M102095_S7	colab. UCAM	Sys-Brain	*Sus scrofa*	Spain
Streptococcus suis	19810_M105628_S32	colab. UCAM	Sys-Brain	*Sus scrofa*	Spain
Streptococcus suis	19808_M105552_S30	colab. UCAM	Sys-Brain	*Sus scrofa*	Spain
Streptococcus suis	19819_M106728_S41	colab. UCAM	Sys-Brain	*Sus scrofa*	Spain
Streptococcus suis	19818_M106471_S40	colab. UCAM	Sys-Brain	*Sus scrofa*	Spain
Streptococcus suis	12RC1 (S12)	JANILI000000000	Tonsil	*Sus scrofa*	Spain
Streptococcus suis	15RC1	JANILJ000000000	Tonsil	*Sus scrofa*	Spain
Streptococcus suis	27RC1	JANILK000000000	Tonsil	*Sus scrofa*	Spain
Streptococcus suis	28RC1	JANILL000000000	Tonsil	*Sus scrofa*	Spain
Streptococcus suis	29RC1	JANILM000000000	Tonsil	*Sus scrofa*	Spain
Streptococcus suis	120RC1	JANILN000000000	Tonsil	*Sus scrofa*	Spain
Streptococcus suis	198RC1	JANILO000000000	Tonsil	*Sus scrofa*	Spain
Streptococcus suis	23QC2CO	JANILP000000000	Tonsil	*Sus scrofa*	Spain
Streptococcus suis	P1/7	AM946016.1	NA	NA	Sanger Institute
Streotococcus parasuis	4253	GCF_004283785.1	NA	cow	University of Zurich
Streotococcus porcinus	NCTC10925	GCF_901553735.1	Tonsil	porcine	Wellcome Sanger Institute
Streotococcus intermedius	NCTC11324	GCF_900475975.1	NA	NA	Wellcome Sanger Institute

Streotococcus porci	DSM 23759	GCF_000423765.1	Heart of a pig with pericarditis	porcine	DOE Joint Genome Institute
Streotococcus pseudoporcinus	LQ 940-04	GCF_000188035.1	NA	NA	J. Craig Venter Institute
Moraxella atlantae	CCUG 66109	GCF_001679065.1	blood	*Homo sapiens*	Sahlgrenska University Hospital
Moraxella atlantae	CCUG 59586	GCF_001678995.1	blood	*Homo sapiens*	K. Jansson
Moraxella atlantae	CCUG 6415	GCF_002027585.1	blood	*Homo sapiens*	NA
Moraxella atlantae	NCTC11091	GCF_900453305.1	NA	NA	NA
Moraxella atlantae	NBRC 14588	GCF_001591265.1	NA	NA	NA
Moraxella boevrei	DSM 14165	GCF_000379845.1	Nasal cavity of a healthy goat	*Capra hircus*	NA
Moraxella bovis	NCTC9426	GCF_900453265.1	NA	NA	NA
Moraxella bovis	CCUG 2133	GCF_002014975.1	NA	Cattle	NA
Moraxella bovis	Epp63 (300)	GCF_003287015.1	ocular secretion	*Bos taurus*	NA
Moraxella bovoculi	23343	GCF_000988655.1	deep nasopharyngeal swab	*Bos taurus*	NA
Moraxella bovoculi	33362	GCF_000988705.1	deep nasopharyngeal swab	*Bos taurus*	NA
Moraxella bovoculi	22581	GCF_000988615.1	deep nasopharyngeal swab	*Bos taurus*	NA
Moraxella bovoculi	28389	GCF_000988685.1	deep nasopharyngeal swab	*Bos taurus*	NA
Moraxella bovoculi	KZ-1	GCF_013307375.1	eye	cattle	NA
Moraxella bovoculi	57922	GCF_000988725.2	eye swab	*Bos taurus*	NA
Moraxella bovoculi	58086	GCF_000988745.2	eye swab	*Bos taurus*	NA
Moraxella bovoculi	58069	GCF_000988605.1	eye swab	*Bos taurus*	NA

Supplementary Table 2. Continued.

Species	Strain	Accession number	Extra information		
Moraxella bovoculi	237	GCF_000696305.1	cow with pinkeye	*Bos taurus*	J. Angelos
Moraxella canis	324_MCAT	GCF_001064535.1	NA	*Homo sapiens*	UW clinical laboratory
Moraxella canis	304_MCAT	GCF_001064405.1	NA	*Homo sapiens*	UW clinical laboratory
Moraxella canis	HAMBI_2792	GCF_003350015.1	NA	NA	NA
Moraxella canis	CCUG 8415A	GCF_002014965.1	Wound	*Homo sapiens*	NA
Moraxella caprae	NCTC12877	GCF_900453285.1	NA	NA	NA
Moraxella caprae	DSM 19149	GCF_000426885.1	NA	*Capra hircus*	NA
Moraxella catarrhalis	K117	GCF_009378155.1	Hospital isolate from Perth	*Homo sapiens*	NA
Moraxella catarrhalis	CCUG 58286	GCF_001679075.1	Human blood	*Homo sapiens*	Camilla Svensson
Moraxella catarrhalis	CCUG 18283	GCF_001679105.1	Human nasopharynx	*Homo sapiens*	Lund Hospital
Moraxella catarrhalis	AS012766	GCF_010604025.1	lung	*Homo sapiens*	Curetis GmbH
Moraxella catarrhalis	CCRI-195ME	GCF_002080125.1	middle ear	*Homo sapiens*	The Research Institute at Nationwide
Moraxella catarrhalis	N4	GCF_001656505.1	Nose	*Homo sapiens*	Hester Bootsma
Moraxella catarrhalis	C031	GCF_001656385.1	Nose	*Homo sapiens*	Stol
Moraxella catarrhalis	N19	GCF_001656205.1	Nose	*Homo sapiens*	Hester Bootsma
Moraxella catarrhalis	FDAARGOS_304	GCF_002984125.1	Nose of a healthy pediatric carrier in	*Homo sapiens*	ATCC < BW Catlin < S Cary < Univ. Maryland
Moraxella catarrhalis	MC6	GCF_003443955.1	oropharynx	*Homo sapiens*	Daria Augustyniak
Moraxella catarrhalis	Z18	GCF_001656255.1	Pharynx	*Homo sapiens*	Hester Bootsma
Moraxella catarrhalis	FDAARGOS_213	GCF_002073215.2	Resident flora of the nasopharynx	*Homo sapiens*	USAMRIID

Moraxella catarrhalis	A6	GCF_009378265.1	saliva frozen stocks	*Homo sapiens*	NA
Moraxella catarrhalis	46P58B1	GCF_003971325.1	sputum	*Homo sapiens*	Univerty at Buffalo, T. Murphy
Moraxella catarrhalis	5P54B2	GCF_003985315.1	sputum	*Homo sapiens*	Univerty at Buffalo, T. Murphy
Moraxella catarrhalis	MC8	GCF_003443975.1	throat	*Homo sapiens*	Daria Augustyniak
Moraxella catarrhalis	MC1	GCF_003443915.1	throat	*Homo sapiens*	Daria Augustyniak
Moraxella catarrhalis	ATCC43617a	GCF_009378275.1	Trans-tracheal aspirate from coal miner	*Homo sapiens*	NA
Moraxella caviae	NCTC10293	GCF_900453175.1	NA	NA	NA
Moraxella caviae	3012STDY6981866	GCF_900683425.1	not available	NA	NA
Moraxella cuniculi	NCTC10297	GCF_900637105.1	Oral mucosa	*Rabbit*	NA
Moraxella cuniculi	CCUG 2154	GCF_002015175.1	Oral mucosa	*Rabbit*	NA
Moraxella cuniculi	DSM 21768	GCF_900156515.1	NA	NA	NA
Moraxella equi	NCTC11012	GCF_900453335.1	NA	NA	NA
Moraxella lacunata	NCTC10359	GCF_900453205.1	NA	NA	NA
Moraxella lacunata	NCTC7911	GCF_900453245.1	NA	NA	NA
Moraxella lincolnii	CCUG 9405	GCF_002014765.1	Human nasopharynx	*Homo sapiens*	K. Lincoln, PHLS
Moraxella macacae	408225	GCF_000320365.1	nasal swab	*Macaca fascicularis*	NA
Moraxella nonliquefaciens	CCUG 60284	GCF_001679155.1	Human cornea	*Homo sapiens*	S. Skovbjerg
Moraxella nonliquefaciens	CCUG 47514	GCF_001679125.1	Human cornea	*Homo sapiens*	E. Kjellin
Moraxella nonliquefaciens	CCUG 348	GCF_001679005.1	nose swab	*Homo sapiens*	Henriksen S. D.
Moraxella oblonga	NBRC 102422	GCF_001598275.1	NA	NA	NA
Moraxella osloensis	YV1	GCF_009867135.1	activated sludge	NA	NA
Moraxella osloensis	CCUG 350	GCF_001553955.1	cerebrospinal fluid	*Homo sapiens*	NA

Supplementary Table 2. Continued.

Species	Strain	Accession number	Extra information		
Moraxella osloensis	KMC41	GCF_002355615.1	cotton	NA	NA
Moraxella osloensis	MOXF1	GCF_005518075.1	culture contaminant	NA	Joe Tully ex Helga Kirchhoff
Moraxella osloensis	NCTC10465	GCF_900453115.1	NA	NA	NA
Moraxella osloensis	YHS	GCF_002752755.2	skin swab	*Homo sapiens*	NA
Moraxella osloensis	KSH	GCF_002752795.2	skin swab	*Homo sapiens*	NA
Moraxella osloensis	TT16	GCF_002786455.2	skin swab	NA	NA
Moraxella osloensis	NP7	GCF_002753715.1	skin swab	*Homo sapiens*	NA
Moraxella ovis	199/55	GCF_001636015.1	case of pinkeye	*Bos taurus*	NA
Moraxella ovis	NCTC11227	GCF_900453105.1	NA	NA	NA
Moraxella ovis	NCTC11969	GCF_900453255.1	NA	NA	NA
Moraxella ovis	NCTC11019	GCF_900453155.1	NA	NA	NA
Moraxella pluranimalium	CCUG 54913	GCF_002014825.1	NA	*Sus scrofa domestica*	A. I. Vela, Complutense University of
Moraxella porci	CCUG 54912	GCF_002014855.1	Brain of a pig with meningitis	*Sus scrofa domestica*	Virginia Aragon, Centre for Research into
Moraxella sp.	HMSC061H09	GCF_001809255.1	sputum	*Homo sapiens*	NA
Moraxella sp.	RCAD0137	GCF_002894025.1	liver	*duck*	Research Center of Avian Diseases, RCAD
Moraxella sp.	VT-16-12	GCF_002224245.2	saliva	*Canis lupus familiaris*	M. Vecherkovskaya
Moraxellaceae bacterium	NA	GCF_902386075.1	human gut	NA	NA
Moraxellaceae bacterium	17A	GCF_902506215.1	NA	NA	NA

Moraxellaceae bacterium	AER2_44_116	GCF_006546595.1	enrichment culture	NA	NA
Moraxellaceae bacterium	HYN0046	GCF_003351745.1	fresh water	NA	NA
Moraxella porci	LG6-2	NA (IRTA)	nose	*Sus scrofa*	IRTA
Moraxella porci	VL15	NA (IRTA)	nose	*Sus scrofa*	IRTA
Moraxella porci	LJ451	NA (IRTA)	nose	*Sus scrofa*	IRTA
Rothia nasimurium	R63	GCA_928382245.1	Tonsil	*Sus scrofa*	Spain
Rothia nasimurium	R652	GCA_928381525.1	Tonsil	*Sus scrofa*	Spain
Rothia nasimurium	R66	GCA_928381515.1	Tonsil	*Sus scrofa*	Spain
Rothia nasimurium	R67	GCA_928381395.1	Tonsil	*Sus scrofa*	Spain
Rothia nasisuis	R69	GCA_928381405.1	Tonsil	*Sus scrofa*	Spain
Rothia nasimurium	R123	GCA_928381615.1	Tonsil	*Sus scrofa*	Spain
Rothia nasimurium	R124	GCA_928381655.1	Tonsil	*Sus scrofa*	Spain
Rothia nasisuis	R141	GCA_928390195.1	Tonsil	*Sus scrofa*	Spain
Rothia nasisuis	R152	GCA_928392805.1	Tonsil	*Sus scrofa*	Spain
Rothia aerolata	R159	GCA_928381445.1	Tonsil	*Sus scrofa*	Spain
Rothia nasisuis	R196	GCA_928392685.1	Tonsil	*Sus scrofa*	Spain
Rothia nasimurium	R206	GCA_928381485.1	Tonsil	*Sus scrofa*	Spain
Rothia nasimurium	R213	GCA_928381465.1	Tonsil	*Sus scrofa*	Spain
Rothia nasimurium	2QC2O2	GCA_928381455.1	Tonsil	*Sus scrofa*	Spain
Rothia nasimurium	19QC2O2	GCA_928383475.1	Tonsil	*Sus scrofa*	Spain
Rothia nasimurium	28QC2CO	GCA_928381425.1	Tonsil	*Sus scrofa*	Spain
Rothia nasuis	R31	GCA_928382305.1	Tonsil	*Sus scrofa*	Spain
Rothia aeria	JCM 11412	GCF_002355935.1	NA	NA	NA
Rothia aeria	C6D	GCF_000763765.1	sputum	*Homo sapiens*	NA
Rothia aeria	C6B	GCF_000763685.1	sputum	*Homo sapiens*	NA
Rothia aeria	NCTC10207	GCF_900637985.1	not available: to be reported later	NA	NA

Supplementary Table 2. Continued.

Species	Strain	Accession number	Extra information		
Rothia aeria F0184	F0184	GCF_000479025.1	oral cavity - mouth	*Homo sapiens*	NA
Rothia aeria F0474	F0474	GCF_000258205.1	biological product [ENVO:02000043]	*Homo sapiens*	NA
Rothia dentocariosa	694_RDEN	GCF_001066935.1	NA	*Homo sapiens*	UW clinical laboratory
Rothia dentocariosa	OG2-2	GCF_002554715.1	kefir	NA	Sonja Blasche
Rothia dentocariosa	OG2-1	GCF_002276695.1	kefir	NA	Sonja Blasche
Rothia dentocariosa	NCTC10917	GCF_900455895.1	NA	NA	NA
Rothia dentocariosa	1234_RDEN	GCF_001061305.1	NA	*Homo sapiens*	UW clinical laboratory
Rothia dentocariosa	UMB0083	GCF_002861025.1	NA	*Homo sapiens*	NA
Rothia dentocariosa	NCTC10918	GCF_900638535.1	Carious teeth	NA	NA
Rothia dentocariosa	316_RDEN	GCF_001064485.1	NA	*Homo sapiens*	UW clinical laboratory
Rothia dentocariosa	FDAARGOS_752	GCF_013267735.1	expectorated sputum	*Homo sapiens*	BEI Resources/ American Type and Culture
Rothia dentocariosa	1C11	GCF_004563855.1	root nodule	*Alder*	NA
Rothia dentocariosa	TB22-02	GCF_012641675.1	Toothbrush	NA	NA
Rothia dentocariosa ATCC 17931	ATCC 17931	GCF_000164695.2	NA	*Homo sapiens*	NA
Rothia dentocariosa M567	M567	GCF_000143585.1	biological product [ENVO:02000043]	*Homo sapiens*	NA
Rothia kristinae	RUTW2-3	GCF_001420025.2	NA	*Tsitsikamma*	Dr Shirley Parker-Nance
Rothia kristinae	ATCC 27570	GCF_004136565.1	skin	*Homo sapiens*	NA
Rothia kristinae	LCT-H5	GCF_001866115.1	Chinese spacecraft Shenzhou X	NA	NA

Rothia kristinae	NCTC11038	GCF_900452535.1	NA	NA	NA
Rothia kristinae NBRC 15354	NBRC 15354	GCF_001570865.1	NA	NA	NA
Rothia mucilaginosa	470_RMUC	GCF_001065115.1	NA	*Homo sapiens*	UW clinical laboratory
Rothia mucilaginosa	UMB0024	GCF_002861015.1	NA	*Homo sapiens*	NA
Rothia mucilaginosa	268_RMUC	GCF_001064265.1	NA	*Homo sapiens*	UW clinical laboratory
Rothia mucilaginosa	NUM-Rm6536	GCF_001548235.1	plaque	*Homo sapiens*	NA
Rothia mucilaginosa	207_RMUC	GCF_001063995.1	NA	*Homo sapiens*	UW clinical laboratory
Rothia mucilaginosa	DE0531	GCF_007666515.1	environmental	NA	NA
Rothia mucilaginosa	FDAARGOS_369	GCF_002386365.1	Blood	*Homo sapiens*	George Washington University
Rothia mucilaginosa ATCC 25296	ATCC 25296	GCF_000175615.1	biological product [ENVO:02000043]	*Homo sapiens*	NA
Rothia mucilaginosa DY-18	DY-18	GCF_000011025.1	NA	NA	NA
Rothia mucilaginosa M508	M508	GCF_000231235.1	biological product [ENVO:02000043]	*Homo sapiens*	NA
Rothia nasimurium	E1706032	GCF_014217215.1	brain	duck	Miaoli Wang
Rothia nasisuis	PT-32	GCF_002087015.1	palatine tonsil	*Sus scrofa*	Rogier Gaiser
Rothia sp	HMSC036D11	GCF_001814205.1	bronch wash cf	*Homo sapiens*	NA
Rothia sp	HMSC058E10	GCF_001809415.1	bronchoalveolar lavage	*Homo sapiens*	NA
Rothia sp	HMSC061C12	GCF_001815755.1	throat	*Homo sapiens*	NA
Rothia sp	HMSC061D12	GCF_001836735.1	sputum	*Homo sapiens*	NA
Rothia sp	HMSC061E04	GCF_001813685.1	sputum	*Homo sapiens*	NA
Rothia sp	HMSC062F03	GCF_001836905.1	sputum	*Homo sapiens*	NA
Rothia sp	HMSC062H08	GCF_001810775.1	sputum	*Homo sapiens*	NA

Supplementary Table 2. Continued.

Species	Strain	Accession number	Extra information		
Rothia sp	HMSC064D08	GCF_001809525.1	sputum	*Homo sapiens*	NA
Rothia sp	HMSC064F07	GCF_001837265.1	sputum	*Homo sapiens*	NA
Rothia sp	HMSC065B04	GCF_001812285.1	sputum	*Homo sapiens*	NA
Rothia sp	HMSC065C03	GCF_001837295.1	sputum	*Homo sapiens*	NA
Rothia sp	HMSC065C12	GCF_001812085.1	sputum induced	*Homo sapiens*	NA
Rothia sp	HMSC065D02	GCF_001815285.1	sputum induced	*Homo sapiens*	NA
Rothia sp	HMSC065D09	GCF_001815155.1	bronchoalveolar lavage	*Homo sapiens*	NA
Rothia sp	HMSC065G12	GCF_001811455.1	sputum induced	*Homo sapiens*	NA
Rothia sp	HMSC066G02	GCF_001813145.1	sputum	*Homo sapiens*	NA
Rothia sp	HMSC066H02	GCF_001814995.1	bronchial washing	*Homo sapiens*	NA
Rothia sp	HMSC067H10	GCF_001810635.1	bronchial washing	*Homo sapiens*	NA
Rothia sp	HMSC068E02	GCF_001813565.1	nasal	*Homo sapiens*	NA
Rothia sp	HMSC069D01	GCF_001810135.1	sputum (induced)	*Homo sapiens*	NA
Rothia sp	HMSC071B01	GCF_001808945.1	sputum(induced)	*Homo sapiens*	NA
Rothia sp	HMSC071C12	GCF_001813895.1	tracheal aspirate	*Homo sapiens*	NA
Rothia sp	HMSC072B03	GCF_001809565.1	sputum (induced)	*Homo sapiens*	NA
Rothia sp	HMSC075F09	GCF_001810515.1	throat	*Homo sapiens*	NA
Rothia sp	HMSC078H08	GCF_001808955.1	tracheal aspirate	*Homo sapiens*	NA
Rothia sp	HMSC08A08	GCF_001808385.1	NA	*Homo sapiens*	NA
Rothia sp	HSID18067	GCF_003989445.1	nasal lavage	*Homo sapiens*	NA
Rothia sp	HSID18069	GCF_003989155.1	nasal lavage	*Homo sapiens*	NA
Rothia sp	ND6WE1A	GCF_001683935.1	single strain isolated from microbial	NA	NA
Rothia terrae	LMG 23708	GCF_012396615.1	wasteland soil	environmental	NA

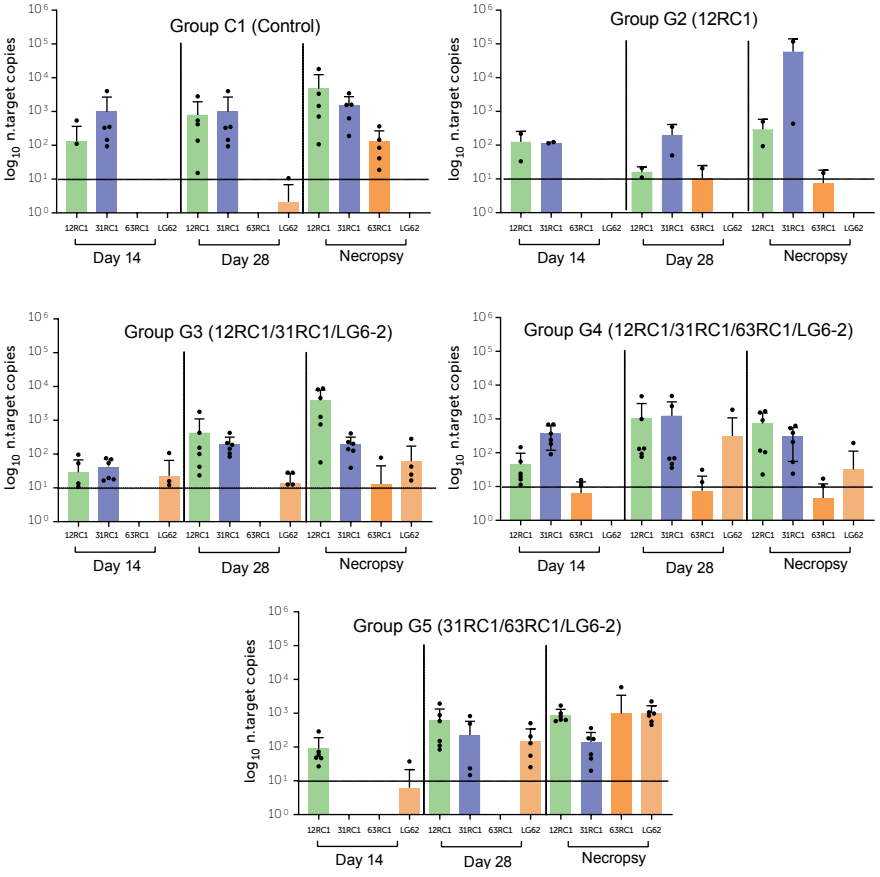
Supplementary Table 3. qPCR primers and probes used to to quantify the candidate probiotics

Isolate ID		Sequence 5'-->3	Amplicon length
Conventional PCR primers to generate DNA template fragments the four selected isolates:			
S12	S12F_external	AAAGCAAAATCGTTGATTCTCGA	551
	S12R_external	GGTTACCTCCAATGTCATTAAAGTGA	
R63	R63F_external	ACACACGGCCACACAAAGTA	584
	R63R_external	AAATGAACCCAGGCGTCACA	
R31	R31F_external	AGAGCTAGTGCGTTCTGCTG	569
	R31R_external	CTACCGGATCTAAGGCGCTC	
LG6-2	LG62F_external	GGAGGTGTCAGATGATTGAGCA	555
	LG62R_external	AGCGCATCAATATCACCTGTG	
qPCR primers and TaqMan probe to quantify the four selected isolates:			
	S12_Fw	GCTCTTCAGAAATTTATGG	
S12	S12_Probe	TAGCCCAACTGAATCAGGTACACC	107
	S12_Rev	CTCTGGAAGATTGGAATC	
	R63_Fw	CTGCTCACACAAAGGATG	
R63	R63_Probe	CCGGCGCTATGACCATCGGT	
	R63_Rev	CAGCGTGACCCTAAAATC	90
	R31_Fw	GCTCTCTATCGTCTCATA	
R31	R31_Probe	CAGGCCAGCTTCACTGTCTATACT	90
	R31_Rev	CTGGGGTAGAAGATAATTTC	
	LG62_Fw	GCCAAAGATGATGAAATG	
LG6-2	LG62_Probe	ACGGCTCAAGATACATCGCTTCATA	92
	LG62_Rev	GGCAATTCGTTAAAATCC	
qPCR primers and TaqMan probe to quantify the S. suis detection:			
	16S_Fw	GAGTGAAGAAGGTTTTCG	
S12	16S_Probe	TTCCACTCTTCTCACAGTTCTTCTCTT	92
	16S_Rev	CTGGTAAGATACCGTCAA	
	VM1_Fw	GAGAGATTGGTCATACAGTA	
R63	VM1_Probe	TCCACATTCACAAGAAGGTCCGT	92
	VM1_Rev	CCAAGTATCAGAAATATAAGTTTG	

Supplementary Table 4. Strains used as control for the qPCR specificity assays.

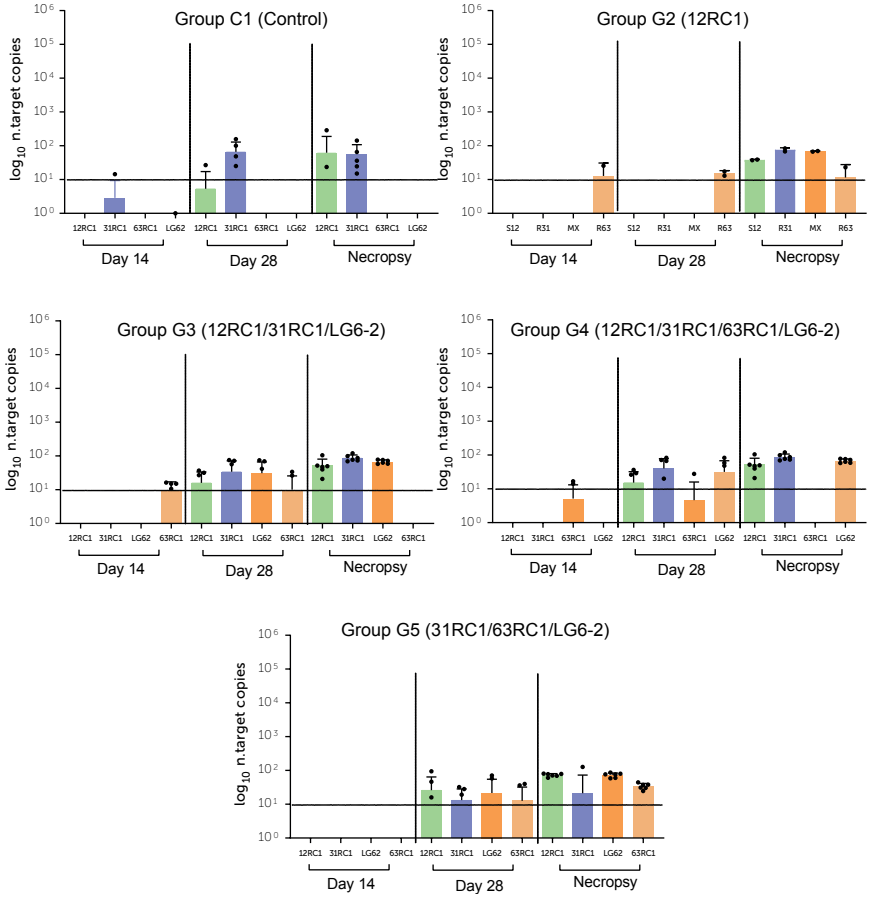
Species	Strain	Source	qPCR control
Rothia nasisuis	R31	Tonsil swab	**Positive**
Rothia nasimurium	R66	Tonsil swab	Negative
Rothia nasimurium	R69	Tonsil swab	Negative
Rothia nasimurium	R63	Tonsil swab	**Positve**
Rothia nasisuis	R141	Tonsil swab	Negative
Moraxella pluranimalium	DSM 22804	Nasal swab	Negative
Moraxella porci	LG6-2	Nasal swab	**Positive**
Moraxella porci	AR10	Nasal swab	Negative
Moraxella porci	GM3	Nasal swab	Negative
Moraxella porci	VL15	Nasal swab	Negative
Moraxella porci	LJ451	Nasal swab	Negative
Streptococcus suis	S12	Tonsil swab	**Positive**
Streptococcus suis	P1/7	Tonsil swab	Negative
Streptococcus suis	S15	Tonsil swab	Negative
Streptococcus suis	S26	Tonsil swab	Negative

A. Tonsil samples

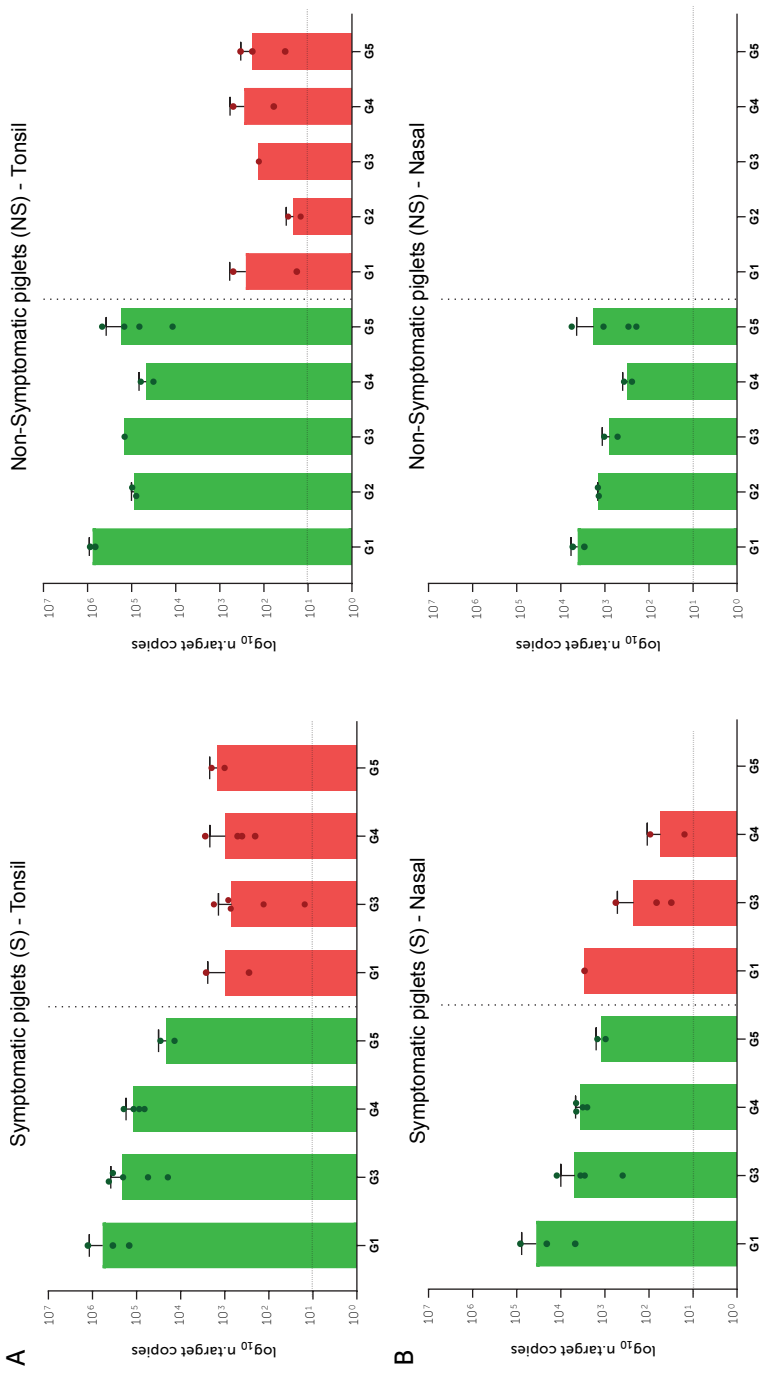


Supplementary Figure 1. qPCR quantification of the four candidate probiotics per intervention group (G2-G5) and control (C1) in tonsil **(A)** and nasal samples **(B)**.

B. Nasal samples



Supplementary Figure 1. qPCR quantification of the four candidate probiotics per intervention group (G2-G5) and control (C1) in tonsil **(A)** and nasal samples **(B)**.



Supplementary Figure 2. Quantification per intervention group (G2-G5) and control (C1) of total amount of *S. suis* and VM1-positive *S. suis* in tonsil (A) and nasal samples (B).

VI

General discussion

The overall goal of the thesis research was to further our knowledge and understanding of *S. suis* interactions with the palatine tonsil and its associated microbiota in the context of colonisation resistance, with the prospect of identifying NGPs that could antagonise colonisation by pathogenic *S. suis*. The sections below discuss the contribution of the main research findings to our understanding of pathogenesis of *S. suis*, the core taxa of the tonsil microbiota and potential to use tonsil isolates to modulate the development of the microbiota and antagonise *S. suis* in the first weeks of life. The concluding section discusses the challenges and opportunities for innovation and future research based on the thesis.

The porcine palatine tonsils as portal of entry for pathogenic *S. suis*

The surface of the porcine palatine tonsils are invaginated by deep crypts sounded by the lymphoid nodules containing germinal centres, as well as blood and lymphatic vessels (1). The lymphoid tissue and crypts contain myeloid cells which form the first line of defence against translocating pathogens and commensal microbiota-derived microbes (2). *S. suis* can naturally being found in the upper respiratory tract, the genital and intestinal tracts of pigs (3). Many studies have reported the isolation of pathogenic isolates *S. suis* from porcine palatine tonsil swabs from healthy and diseased pigs suggesting the palatine tonsils are a portal for *S. suis* to enter the body and disseminate to cause sepsis and meningitis (4, 5). The intestinal route has also been proposed as possible entry site for *S. suis* due to increased colonization of *S. suis* after weaning. Studies has investigated the intestinal route by inoculation directly in the intestine or oral capsule-mediated delivery of virulent strains of *S. suis* into the small intestine (6, 7). However, additional factors as the inoculum size, stress conditions, and the low proportion of challenged animals which developed invasive disease in those studies shows that the translocation from the intestine seems to be possible but not a very effective route of infection (8). Notwithstanding the possibility of different points of entry into the body, we focused on studying the palatine tonsils.

The anatomic position of the palatine tonsils close to the oral cavity and the nasal airways raises several questions, including how the tonsils contribute to mucosal immunity, and what role microbiota play in colonisation resistance to pathobionts and obligate pathogens. In **chapter II** we reported on the structural complexity of the palatine tonsils, presence of different subsets of macrophages or monocytes and microorganisms in tonsillar biofilm and within the crypts (Figure 1).

By histology we showed that Gram-positive bacteria are present deep in the crypts of the palatine tonsils. The crypt content appears to contain bacteria, cell debris and possibly live intraepithelial lymphocytes or myeloid phagocytes which may

have undergone trans-epithelial migration. Deep in the crypt the stratified epithelium becomes a single-cell layer and lacks the basolateral layer of extracellular matrix otherwise known as the 'basal laminar' commonly found in epithelial tissue. Here, we observed bacteria inside innate cells lining the crypt and within the interstitial areas. Viable *S. suis* was recovered from the tonsillar macrophages isolated from the tonsillar lymphoid tissue, suggesting that virulent *S. suis* may survive in the tonsil, enter the efferent lymphatics and ultimately, the bloodstream, to cause systemic infection.

The recent finding that *Streptococcus pneumoniae* can replicate in CD169+ macrophages in the spleen of mice led us to investigate the presence of this macrophage subset in the porcine tonsil. Using the anti-human CD169 antibody we detected these macrophages in high abundance in the porcine tonsil. Most were surrounding the germinal centres consistent with their proposed role in capturing antigens for presentation to T cells in concert with dendritic cells (11). However, CD169+ macrophages were also found near to the epithelium lining the crypt where they may have been be recruited by chemokines. As CD169+ macrophages are reported to have weak killing activity against bacteria, virulent *S. suis* isolates might survive or even replicate in CD169+ macrophage. Thus CD169+ macrophages may function as a reservoir for *S. suis* before entering the bloodstream and causing clinical disease, similar to what has been shown in *S. pneumoniae* (12).

CD169 is a sialic-acid-binding immunoglobulin-like lectins (siglec) receptor (13) which would bind to the sialic acid containing capsular polysaccharides produced by some pathogenic serotypes of *S. suis,* for instance serotype 2 and 14, which is commonly associated with invasive disease in pigs and humans Therefore we hypothesise that intracellular survival and replication of *S. suis* in CD169+ macrophages in the tonsil may increase the likelihood of transport through the efferent lymphatics to the subclavian vein and bloodstream (14). However, several questions remain to be addressed:

Does *S. suis* indeed replicate and survive after phagocytosis by CD169+ cells?
Does *S. suis* escape from the phagosome ?
Does *S. suis* phagocytosis lead to apoptosis or pyroptosis of CD169 macrophage?

The novel evidence we presented here for an intracellular phase of *S. suis* within CD169+ macrophages in **Chapter II** is relevant for understanding the initial steps in the pathogenesis of S. suis invasive disease and resistance to antibiotic therapy (Figure 1).

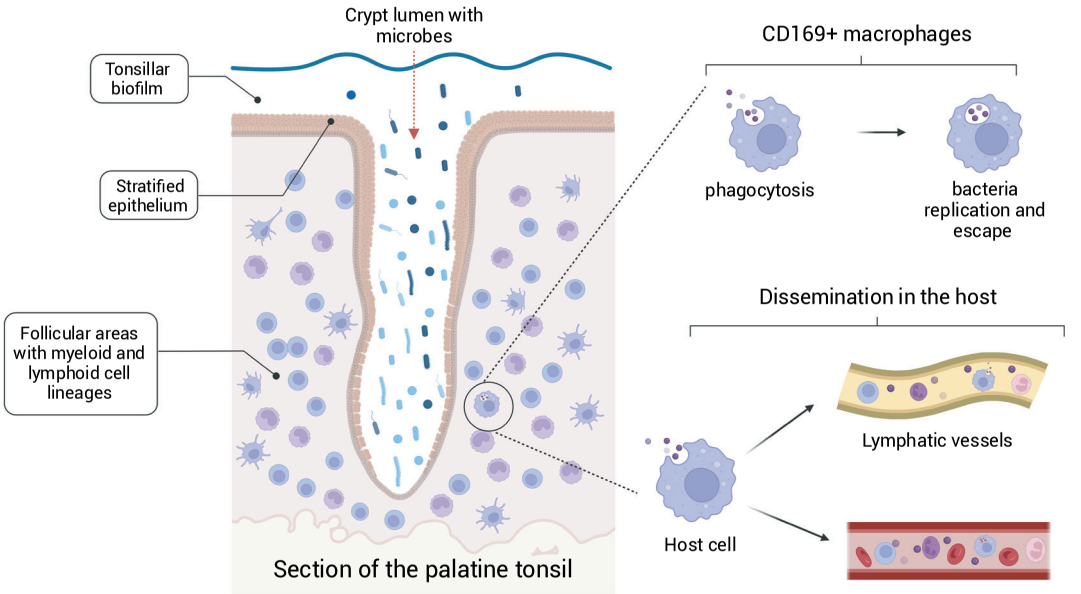


Figure 1. Hypothesis for role of CD169+ macrophages as reservoirs of replicating *S. suis* and the dissemination of *S. suis* from the tonsil through lymphatics or blood vessels. Multiple lineages of myeloid and lymphoid cells have been identified in the tonsils, including subsets of conventional dendritic cells (cDC) (cDC1s and cDC2s), plasmacytoid dendritic cells (pDCs), as well as a monocytes, macrophages and lymphocytes (T and B cells) expressing several cell surface markers (2, 9). We found that the porcine lymphoid tissue contains a relatively large number of CD169+ macrophages as well as CD163+ macrophages or monocytes and dendritic cells. *Streptococcus pneumoniae* has been shown to be captured by splenic CD169+ macro- phages, in which they can replicate intracellularly and ultimately disseminate from the spleen back into the blood stream to cause sepsis (10). We hypothesise that pathogenic *S. suis* entering the tonsil lymphoid tissue is phagocytosed by CD169+ macrophages, replicates intracellularly causing macrophage death due to suilysin secretion which leads to release of *S. suis*. *S. suis* accumulating in the tonsil might then disseminate directly through blood vessels in the tonsil or via the efferent lymphatics back to the bloodsteam, potentially attached to or inside host immune cells.

The tonsil microbiota and colonization resistance

The use of antibiotics causes collateral damage to the microbiota: reduction of functional diversity, and overall disruption of the homeostatic and complex network of the microbiome which may lead to loss of microbiome homeostasis and dysbiosis, which in turn may lead to the host's susceptibility to diseases (Figure 2) (15, 16). There are several studies showing loss of homeostasis of the native microbiome and the host's response to a dysfunctional or dysbiotic ecosystem can be associated with disease (17-19). For example, children receiving antibiotic in the first 3 years of life have less stable and less diverse bacterial of the faecal microbiota and more frequent bacterial infections (20). In piglets with later weaning (42days of age) lead to higher faecal microbial diversity and less incidence of enteric diseases, poor intestinal health, and intestinal inflammation, as well as better growth and weight gain (21). Recently piglets (21days old) were used to study the effect of antibiotic administration on gut microbiota diversity during consecutive 14days. As expected, pigs receiving antibiotic treatment had lower richness and diversity of species in faecal microbiota and increased abundance of opportunistic pathogens after 2 weeks. Furthermore the metabolites promoting intestinal immune homeostasis were increased or decreased based on the antibiotic treatment or not (22).

There is considerable interest in maintaining or improving intestinal microbiota diversity and functionality to provide colonisation resistance against pathogens, and promote intestinal homeostasis. For instance, faecal microbiota transplantation has been reported to confer substantial reductions of recurrent *Clostridium difficile* in adults, with cure rates approaching 90% (23). The introduction of symbionts or core species is considered one approach to promote beneficial host-microbe interactions, including colonisation resistance against pathogens and pathobionts.

Research in this thesis was aimed at identifying isolates which are prevalent and abundant in the pig tonsil microbiota which could antagonise pathogenic *S. suis* though direct production of antimicrobials or indirectly provide colonisation resistance through modulation of the microbiota. Our study on more than 100 tonsil swabs showed the porcine microbiota to be extensive and diverse with high abundance of *Actinobacillus, Streptococcus* and *Moraxella*. Based on prevalence and abundance in the tonsil microbiota across different countries and farms, we propose 14 genera to be core species, with potentially important interactions in the ecology of this microbial community. These were selected from the collection of cultivatable isolates from tonsil microbiota characterised in **Chapter III**. This collection consists of over 500 isolates representing 60 species across 23 genera and four phyla, which is as far as we know is the most comprehensive collection of tonsil associated isolates reported. Analysis of full genome sequences of representative taxa from

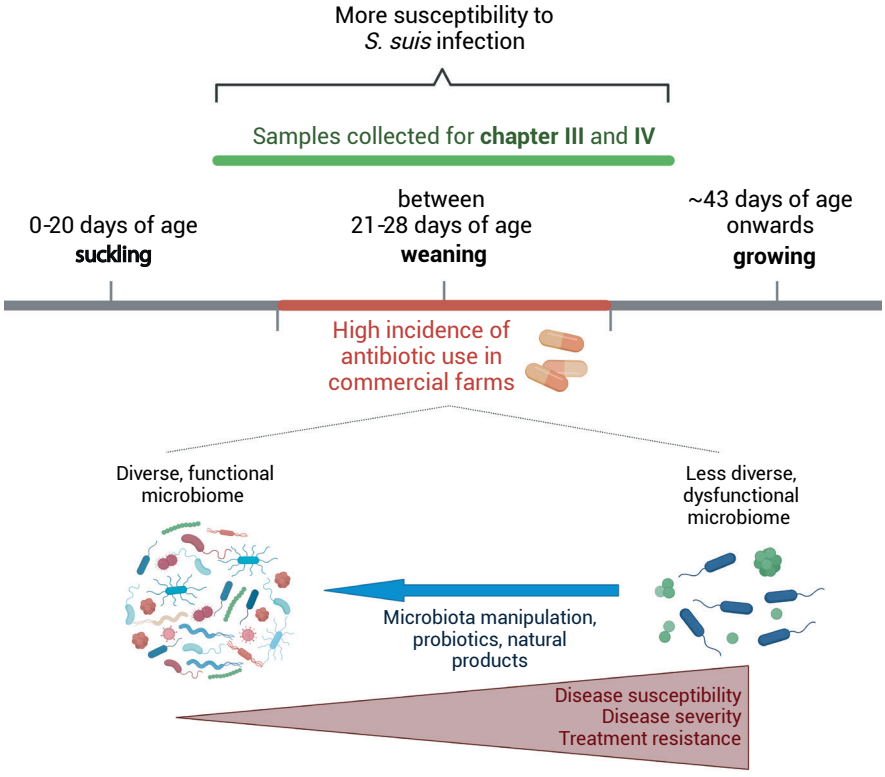


Figure 2. Scheme showing piglet ages and antibiotic use for samples collected in this study. Microbiome dysbiosis caused by antibiotics increases the risk of infection and intestinal problems. Possible interventions to restore a diverse microbiota are shown under the blue arrow.

these 14 genera revealed numerous biosynthetic gene clusters likely involved in competition and production of antimicrobials for inter-strain and species competition.

Given that *S. suis* infections occur mainly in young piglets during the first 2 weeks after weaning, we hypothesised that it may be possible to manipulate the development of the tonsil microbiota during the first four weeks of life to stimulate innate immunity and promote colonisation resistance. To make a selection of candidates for an *in vivo* study we relied on several other criteria we could assess by *in vitro* assays (**chapters III and IV**). Two assays tested for direct inhibition of *S. suis* growth on agar plates and ability to adhere to tonsil epithelium which we speculated might promote colonisation and persistence in the same niche occupied by *S. suis*. Other criterion used for the selection were to avoid administering

strains carrying antibiotics commonly used to treat bacterial diseases in pigs. Although none of the species selected were obligate pathogens, we avoided isolates with high serum resistance to complement which might increase the risk of opportunistic infections in compromised hosts.

Development of a multi-species microbial inoculum for an *in vivo* challenge study

In **chapter V** we tested the ability of cocktails of the four isolates to promote development of a stable microbial ecosystem and protect against challenge with pathogenic *S. suis*. Unfortunately the experiment suffered substantially from an enteric *E. coli* infection starting on day 2 which meant that 19 out of 44 (43%) piglets with symptoms of infectious diarrhoea had to be removed from the study and the remaining piglets were treated with antibiotics on days 3, 4 and 5 to prevent further problems. Notwithstanding the loss of statistical power, positive influences of commensal isolate colonization on piglet survival and lesion scores in the two piglets that developed disease were observed with group (G5) (Figure 3) which we consider to warrant further study. G5 consisted of one *Moraxella* isolate and two *Rothia* isolates, one of which produces an antibiotic killing *S. suis* (**Chapter V**).

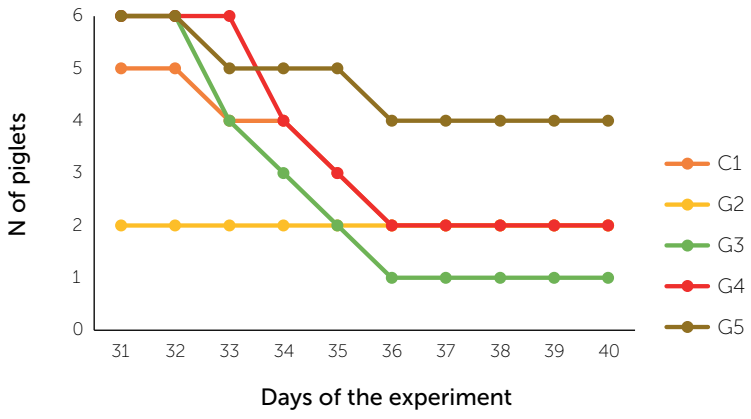


Figure 3. Survival of the piglets after challenge with virulent *Streptococcus suis* P1/7 (serotype 2) at day 30 of life. Piglets had been intranasally and orally inoculated with different combination of commensal strains on day one and day six after birth. Combinations of commensal strains were described in **chapter V;** numbers of piglets per group at day 30 were as follows: C1; five piglets, G2; two piglets, G3; six piglets, G4; six piglets, G5; six piglets. (C) control, (G) group.

The dual inoculation with commensals was not sufficient to protect the inoculated piglets against *S. suis* infection. However, considering that the respective isolates were sampled from the microbiota of healthy piglets and present relevant features (**chapter V**), they may exert positive functions toward the host apart from direct protection against disease-associated bacteria. For example, a recent study has shown that administration of probiotics in neonates contributed to the restoration of gut microbiota after antibiotic exposure (24). Such effects are reminiscent of the effects expected from therapy using so called next generation probiotics, natural (abundant) gut bacterial strains that enhance gastrointestinal immunity, maintain intestinal barrier integrity and improve other host conditions (25). Although limited information was obtained in this thesis regarding potential roles of investigated isolates *in vivo*, characteristics of some strains and warrant further investigation (Table 1).

The impact and the biological effect of isolates introduced in a more or less established microbial community is directly determined by metabolic stimuli received from other resident isolates of the same and other species (26) as well as from the host and its nutrition, and direct competition through natural product. Rationally designed "cocktails" containing a sufficient number of metabolically redundant isolates provide the possibility to get some measure of the stability of the community, composition and even gene expression (27). However, introducing a low-complexity cocktail containing only a few strains with nonredundant metabolic capacities may have insufficient capacity to beneficially impact microbial symbiosis within the niche, unless an early intervention is made. Probiotic applications carried out during the so-called "window of opportunity" in early life may modulate niche colonization and development of homeostatic microbiota and have long-term influence on proper development of the immune system (28). It is not known if single species can be efficiently introduced in a microbial community in order to successfully colonise and persist in a niche although recent studies suggest this is feasible depending on age and the frequency of administration (29). Introduction of a defined community containing less than 10 strains has been shown to promote microbiome homeostasis resulting in lower inflammatory responses in a controlled mouse model (30) and promoting efficient conversions of gut dietary fibres into short chain fatty acids (31). Recently, a study involving humans and a controlled mouse infection model demonstrated that use of a more complex community consisting of 104 selected strains promoted robust colonization resistance against *E. coli* and establishment of a phenotypically similar human faecal community in the gut microbiome of germ-free mice (32).

Table 1. Main characteristics of the candidate new generation probiotics identified in this thesis

Genera/Species/Isolate	Correlation with *S. suis*	Features	Chapter
Actinobacillus commensal species	possible positive correlation in a symbiotic microbiota of healthy piglets	Overall abundant and prevalent in the microbiota	3
Corynebacterium kozikiae 76QCO2		Producer of a stable nontoxic lanthibiotic able at low concentrations to inhibit *S. suis* and other Gram-positive bacteria	3
Rothia nasisuis isolates		Producer of valinomycin able to inhibit *S. suis* and other Gram-positive bacteria	3,4
Lactobacillus species		Prevalence and relative abundance higher in the microbiota of non-severe infected piglets	5
Moraxella porci isolates		Genera with high relative abundance in the microbiota and presence in the cocktail of the group G5 with higher survival	5
Alloprevotella genus	possible correlation with a dysbiotic microbiota during *S. suis* infection	Relative abundance higher in the microbiota of piglets which developed severe *S. suis* infection	5
Porphyromonas genus		Relative abundance higher in the microbiota of piglets which developed severe *S. suis* infection	5

Rational strain selection to develop complex microbial communities allows one to address the functional capacities of a community in different niches. Recent studies have stimulated efforts to build complex communities by directed evolution, engineering, and reintroducing combinations of microbes to understand the fundamental biology of host-microbe interactions as well as for environmental applications including biocontrol, biofertilization, and biostimulation (33-35). Taken together, the available knowledge of the microbiota in health and disease combined with recent technological advances may enable the development of, for instance, promising and sustainable alternatives to conventional faecal microbiota transplantations (using faecal samples from unrelated donors) in treating disorders as inflammatory bowel disease (36). Design of stable and controllable (synthetic) microbial consortia might bring several positive outcomes, for instance, increasing the efficiency of biogas production from waste (37), safe strategies to increase sustainability in agriculture (34) or improve and modulate the ability to convert complex mixed substrates into valuable products in a bioreactor (38).

The *in vivo* study reported in **Chapter V** provided valuable insights on early colonization and establishment of commensal *S. suis* in tonsillar and nasal microbiota. We compared composition and relative abundance of the microbial community in the piglets with and without symptoms of *S. suis* infection and found that strains from the genera *Alloprevotella, Porphyromonas* and *Moraxella* had been present at higher abundance in diseased animals (Table 1). We hypothesised that such strains might have promoted increased tonsillar microbiota colonization by virulent *S. suis* isolates, and that future *in vitro* research on co-inoculations of disease-associated isolates from these three genera and virulent *S. suis* strains might show unexpected metabolic interactions.

A Bacterial Genome and Culture Collection of Tonsil Microbiota in Weaned Piglets

In **Chapter III** we assembled a comprehensive resource of pig tonsillar species comprising over 500 isolates representing 60 species across 23 genera and four phyla. The whole genome sequences (WGS) of 89 tonsillar isolates together with genomic and a biochemical description of two proposed novel species was deposited in open-source databases and archives. This makes an important contribution to the diversity of current microbial data which is heavily biased towards intestinal bacteria. Collections of cultured bacteria from different sites of human, animals and environment have been essential for experimental validation, interpretation and assessing the functional potential of microbiota (39-42). For instance, a culture-based study on female bladder microbiota where approximately two thirds of bacteria had been cultured demonstrated an interconnected urogenital

microbiota community with functional capacities that were distinct from what had been described for human gut microbiota (40). Other studies from animal and human microbiota have also reported valuable findings of the functional potential of microbiota in health and disease, as well as differences between hosts and environmental conditions (41-43). A representative bacterial collection from mice has identified species that are specific to the mouse intestine and using this collection, it was shown that a minimal consortium of 18 strains covered 50–75% of the known functional potential of metagenomes (41). Recently, a collection of over 100 species across nine phyla representing the porcine microbiota identified relevant features in the genomes of newly isolated species such as conserved gene clusters for biosynthesis of antimicrobials and deconjugation of primary bile acids (42). Culturing bacteria isolated from plant and soil ecosystems has also led to the discovery of numerous leads to future applications, including bacteria able to induce plant systemic resistance, modulate hormones, production of natural antibiotics, volatile molecules that modulate microbial interactions and metabolism, and adaptation factors such as (iron) metal-scavenging siderophores (44).

One major limit of culturomics is that it depends on culture media and specific growth conditions. Some species are highly fastidious and it is not always obvious what medium components and growth conditions are optimal. The yet uncultured portion of the microbiota (33% relative abundance) may be cultivable when favourable growth conditions, including essential nutrients are provided. As such, selection and rational decisions which factors allow growth of these organisms in laboratory conditions are critical to improve the culturing of fastidious species. In our study, we used mostly non selective and non-enriched media with manual isolation procedures, and we indeed observed the absence of certain taxa after comparing cultured taxa and their relative abundance to 16S rRNA gene sequence-based relative abundances. Longer periods of incubation, different temperatures, oxygen pressure, as well as use of different dilutions are strategies that have been used to promote the growth of fastidious bacteria. The anatomy of the tonsil and presence of bacteria in the deep invaginations of the tonsil crypts (**chapter II**) suggests that microaerophilic conditions in addition to a more complex medium composition may promote *in vitro* growth of previously uncultured taxa. Lagier et al., for instance, have used 212 different culture conditions and reported more than 900,000 bacterial colonies from which 1,057 bacterial species were isolated and purified, including 531 species that had not been found in the human gut before; the same authors reported 247 new prokaryote species (45). New techniques as single-cell technologies can provide unique insights into the biology and within-species diversity of complex microbiomes at spatial scales (46) that go beyond the more common community dilution-based culturomics techniques used in this thesis.

The data from the cultured fraction of porcine tonsil microbiome has brought new insights and resources for functional studies, such as efforts to reduce infections in the oral cavity via microbiome manipulation, and discovery of new antimicrobials as described in **Chapter III** and **Chapter IV**. Future, studies on larger number of samples, more (diverse) culture conditions, and complementary long-read metagenomic sequence-based analyses such as microbiome signatures to predict disease incidence, could help to elucidate the unexplored activities of members of the native tonsil microbiota.

Uncovering secondary metabolite biosynthetic gene clusters in genome sequences of tonsillar bacteria

As an example of the utility of microbiota culture collections we mined the genomes of a representative species from the microbiota for biosynthetic gene clusters (BGCs). Microbial BGCs contain co-expressed genes encoding diverse types of enzymes and transport mechanisms via which molecules with a large range of biochemical activities may be produced and released into the environment (47, 48). Molecules produced by BGCs are often used for intra- and interspecific signalling in biofilms, host-microbe interactions, and niche colonization and -persistence. Some BGC-produced molecules play direct roles in health and disease, for example, toxins secreted by certain disease-associated *E. coli* strains are produced by BGCs (47, 49, 50). The largest three classes of BGC-derived natural products that are precursors of promising active natural products belong to distinct chemical groups, most often ribosomally synthesized and post-translationally modified peptides (RiPPs), polyketides (PKs) and nonribosomal peptides (NRPSs) (51). These secondary metabolites may serve as chemical cues that promote bacterial colonisation and homeostasis at tonsillar biofilm surfaces (52).

When investigating metagenomic data generated in our group from over 100 genome sequences representing taxa from our porcine tonsillar microbiota samples, *in silico* analysis identified over 990 biosynthetic genes clusters (BGCs), distributed into several classes. Approximately 56% of the identified BGCs belonged to the classes NRPS, PKS and RiPPS. These results highlighted the potential of the tonsil microbiota as a natural reservoir for compounds with antimicrobial activity, further explored in this thesis. The relevance of the oral cavity microbiota as producers of compounds with antimicrobial activity was shown in a human oral microbiome study where 4,915 BGCs were identified across 461 genomes from diverse species(47). Using conventional approaches for drug discovery (isolation of bacteria, culturing and bioactivity screening) described in **chapter III,** we uncovered 79 isolates that were able to inhibit *S. suis* and other common members of the microbiota. Genome sequences were obtained from the different species of interest for genome mining.

This led to the discovery of a novel species of *Corynebacterium* (that we named *C. kozikiae*), producing a variant of the lanthipeptide flavucin that has a potent but narrow-spectrum growth-inhibiting activity against different streptococcal species and *S. aureus*. The purified extract of this small molecule was active at different pH's and high temperatures and was not toxic to mammalian cell lines, features that are attractive for future characterization and antimicrobial applications.

In **Chapter III** and **Chapter IV** other classes of BGCs known to produce bioactive molecules such as terpenes which are known to play significant roles in the host defence or as chemical messengers for conspecifics and mutualists (53). Terpenes and terpenoids are the main bioactive compounds in essential oils and have been comprehensively studied because of their antimicrobial properties and use as food preservatives (54). Another example of relevant class of BGCs is the siderophores which are high affinity iron chelators (55) with over 20 clusters identified in our bacterial genomes. Iron plays essential roles as cofactor in various metabolic processes including growth in the majority of organisms (56). Two pathways, one NRPS dependent and one NRPS independent, are mainly involved in siderophore biosynthesis(57). Some siderophores have additional indirect functions: cephalosporin, a siderophore that forms a complex with extracellular free iron, has bactericidal activity through cell wall synthesis inhibition, at least against multiple clinically relevant multidrug-resistant Gram-negative bacteria (58).

The high abundance of BGCs identified in genomes of species in the tonsil microbiota likely reflects the importance of secondary metabolites in successful colonization of the tonsils (e.g iron acquisition by siderophore) and ecological interactions (antimicrobials for competition). It is important to highlight that BGCs can often be associated with mobile genetic elements (MGEs), suggesting the possible transfer of biosynthetic capacity from commensal species to other unrelated species, including opportunistic pathogens or pathobionts (59). *S. aureus* is one example of opportunistic pathogen that showed relevant antimicrobial activity against *S. suis* in our antagonism screens; and genome mining identified BGCs predicted to produce bioactive secondary metabolites. In addition to *S. aureus,* other staphylococcal isolates have shown inhibitory activity against a diverse range of commensal bacteria and pathogens (49). In staphylococci, most of these BGCs are genetically linked to MGEs and transferred between strains and lineages which supports the idea that secondary metabolites are important for niche colonization and pathogenicity (60). Our findings not only bring insights towards the potential of discovering novel BGCs when sampling less well studied host niches, but also informs on the distribution of different classes of BGCs, conservation across species and metagenomes of the tonsil microbiota species, and their potential functional roles.

Screening whole genome sequences to find biosynthetic gene clusters predicted to produce antibiotic compounds underlying the inhibitory potential of porcine tonsillar microbiota isolates

We found that *Rothia* species were among the most prevalent bacteria in the upper respiratory tract of pigs (**Chapter III**) as reported for the human oral cavity (61). *Rothia* species are members of the Actinomycetota phylum which includes *Streptomyces* a genus which produces over two-thirds of the clinically useful antibiotics of natural origin. To date this species has been little studied except for a recent study reporting the production of the antibiotic ionophore valinomycin by an NRPS in *Rothia nasisuis* (62). Valinomycin is antibacterial, antifungal and active against some viruses and was previously only known to be produced by *Streptomyces*.

In **chapter IV** we reported among others that NRPS clusters are widely present in genome sequences of different *Rothia* species, and that some of these were predicted to produce natural antibiotics that have in-host bioactivity, including (i) sensitizing epithelial cells for efficient innate immune defense, (ii) increase the recruitment of phagocytic cells and (iii) act synergistically with microbiota- or host-derived factors (63). We also isolated strains belonging to the species *Rothia nasisuis*, a species to date only isolated from pigs, that produced valinomycin. Isolates of the species *R. nasimurium*, *R. mucilaginosa* and *R. dentocariosa*, were predicted to produce natural antibiotics with high similarity to enterobactins, siderophores that could be involved in colonization of the oral cavity. *In silico* analyses of *R. nasimurium* genomes identified BGCs encoding NRPS with low similarity to known clusters which might produce novel bioactive compounds (Figure 4A). However, investigating bioactivities of molecules produced by BGCs in the laboratory can be challenging since BGCs are often not-expressed under laboratory conditions. For instance, isolates from the genus *Streptomyces* that is well known for the production of antimicrobial products, do not to express 90% of their BGCs *in vitro* (64). To overcome such limitations, strategies to activate the production of specialized metabolites by bacteria such as *Streptomyces* have been developed and optimized in native and heterologous hosts (64, 65).

We used heterologous expression to further investigate the NRPS of interest identified in **Chapter IV**, by BGC "refactoring", a term for cloning of partial clusters or entire BGCs in the heterologous expression hosts such as *Streptomyces coelicolor* and *Pseudomonas putida*. We predicted cluster regulatory regions with biosynthetic and transport-related genes of the *R. nasimurium* strains and cloned these together with the associated genes under their regulation into the corresponding expression vector (Figure 4A). The NRPS-like BGCs cloning and

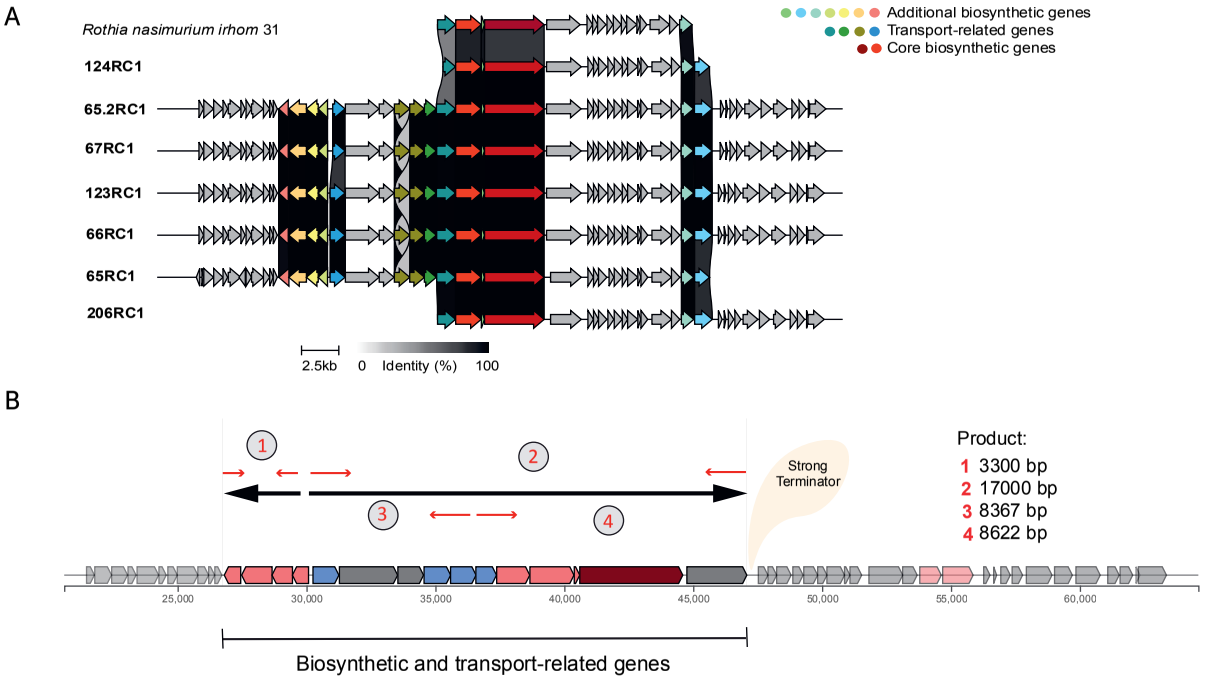


Figure 4. BGCs annotated as NRPSs predicted to produce relevant bioactive compounds and strategy to activate silent BGCs. A) BGCs identified in the genomes of the isolates belonging to the species *R. nasimurium* with conserved genes also found in the genome of *R. nasimurium* strain *irhom* 31 (NCBI accession SPQC01000000). Annotations and functional predictions were *in silico* generated by AntiSMASH. B) Primers designed to amplify NRPS biosynthetic genes for BGC refactoring and heterologous expression. The strategy was based on assembly of multiple PCR fragments spanning BGC biosynthetic genes, together with their respective predicted regulatory genes and terminator sequences. The displayed primers were designed to assemble the region: 1-2 and 1,3 and 4. Size of the displayed clusters is 23kb.

heterologous host transformation strategy involved transfer of large DNA fragments varying between 3300bp and 17Kb (Figure 4B). Although this could promising work could not be finished during the thesis, it will be continued.

WGS analysis and comparative genomics are a useful approach to discover genes across *Rothia* species and isolates, and use of functional gene annotations enabled us to predict correlations between gene presence/absence and colonization of different niches by different *Rothia* species and isolates. Genomic analyses showed that *Rothia* genomes encode a large number of carbohydrate-active enzymes (CAZy) with diverse functions including nitrate-reducing enzymes that were reported to promote human oral health (66). Summarising, WGS analysis of core and accessory genomes from representative *Rothia* strains suggested that the genus contains species with versatile metabolic capacities to adapt to different hosts and environments. Presence of BGCs associated with production of natural compounds with antibiotic activity in some strains and species suggested that specific *Rothia* strains might show antagonistic activity against disease-associated bacteria like virulent *S. suis*, and that it might be worthwhile to investigate probiotic potential of such *Rothia* strains.

Concluding remarks

Our research results and outcomes have societal impact through their contribution to both fundamental and applied science. We showed that *S. suis* is not only a core species of the porcine tonsil microbiota but that it is an early coloniser, establishing itself in the oral cavity in first few days of piglet life. We identified core taxa of the tonsillar microbiota based on prevalence and abundance and showed that the composition of the tonsillar microbiota may be correlated with early-life infections of piglets by virulent *S. suis*. Because of the structural complexity of the upper respiratory tract and its diverse niches, a more systematic approach or even, specific approaches to explore these niches may lead to discovery of taxa that provide some health benefit to piglets, including taxa that produce small molecules that might be further developed into anti-infective or antimicrobial compounds. For instance, the combination of different omics technologies as culturomics, (meta-)genomics, and metabolomics together with *in situ* imaging of specific bacteria could be utilised to obtain an unbiased understanding of microbial interactions in a niche. Further research on the novel BGCs identified in this thesis is warranted due to the urgent need for new antibiotics to combat antimicrobial resistance in human and animal pathogens. The secondary metabolites we showed to be produced *Rothia* species and *C. kozikiae* inhibited multiple pathogens and

based on their known functions in other bacteria, might promote positive microbiota-host interactions. Further experiments on the properties of the bioactive metabolites we discovered such as flavucin IF76 might indeed open doors to development of novel antimicrobial compounds.

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APPENDICES

Summary

Acknowledgements

About the author

List of publication

Overview of Completed Training Activities

Summary

Streptococcus suis is a Gram-positive bacterium which causes meningitis, sepsis, and other diseases in swine. Disease-associated *S. suis* strains are considered part of porcine respiratory disease complex, which is one of the major causes of mortality in piglets. Efforts to reduce the use of antibiotics in livestock and the lack of effective vaccines have led the scientific community to search for more sustainable and applicable solutions to the pork industry worldwide. Antibiotic therapy has decreased the mortality rate caused by infectious diseases and, together with improved sanitation and vaccine administration, has markedly increased human longevity and animal health. But widespread application of antibiotics has led to development and spread of antibiotic resistance, partly driven by overuse of antimicrobials in clinical and agricultural settings. Previous research has shown the promise of using commensal bacteria as producers of natural products and as candidate probiotics in the manipulation of the microbiota and disease preventions and treatments (**Chapter I**). The work described in this thesis focuses on the palatine tonsil microbiota as source for natural bioactive compounds and probiotics candidates to control infections. Moreover, we investigated the role of tonsils as portal entry for *S. suis* infection. Our research has provided insights that can be used to devise strategies to reduce *S. suis* infectious disease problems and avoid spread of antibiotic resistance in microbiota including those communities where relative abundance of *S. suis* is high. Our research outputs also contribute to our understanding of the composition of the tonsillar pig microbiota and the some metabolites that are produced in tonsillar biofilms.

In the first part of our research we investigated the evidence for an intracellular phase in systemic *S. suis* infection of piglets . In **Chapter II** we showed that bacteria present at the intersection of the epithelial lining of the palatine tonsil crypts and within the lymphoid tissue. We identified a considerable number of CD169+ macrophages in the tonsil lymphoid tissue. In addition, myeloid cells with associated *S. suis* bacteria were isolated, suggesting that sequestration by and survival within macrophages and monocytes might contribute to systemic dissemination of bacteria. We propose that virulent strains may be able to persist intracellularly in CD169+ macrophages, and that future research determining whether *S. suis* isolates can survive within tonsillar macrophages and monocytes for an extended period of time.

Chapter III describes the tonsillar microbiota of healthy piglets and isolated commensals which inhibit the growth of *S. suis* via antimicrobial molecules. By investigating the microbiota composition of over 100 samples and plating tonsil swabs on different media and culturing hundreds of different colonies, we identified 518 isolates belonging to four different phyla. We performed pathogen inhibition screenings and *in silico* genome mining to assess the potential to produce antimicrobial compounds, and focused our research on a few bacteria that produced small molecules that might play a role(s) in colonization resistance against pathogenic microbes. We reported that tonsil microbiota include bacteria that produce specialized metabolites that appear to be a relevant but underexplored source for drug discovery and development of antimicrobial products. Our tonsillar bacterial collection provides a resource for discovery and development of next generation probiotics candidates.

In **Chapter IV** we focused on the genus *Rothia*, a prevalent inhabitant of the human and animal upper respiratory tract and oral cavity. We cultured *Rothia* members of the porcine tonsil microbiota and assayed strains for the production of antibacterial compounds with activity against a panel of "target" bacteria that included disease-associated and commensal bacteria. The genomic analyses of 43 newly isolated porcine *Rothia* strains, together with publicly available draft genome sequences of *Rothia* isolates from humans, animals and the environment revealed potential niche-associated colonization mechanisms and metabolic specialization that may be relevant for polymicrobial interactions. All genomes carried biosynthetic gene clusters (BGCs) predicted to produce antibiotic non-ribosomal peptides (NRPs) and other secondary metabolites that may have the potential to modulate ecological interactions and function in niche and host adaptations.

In **Chapter V** four commensal isolates were selected as candidate probiotics based on their abundance in the healthy piglet tonsillar microbiota and *in vitro* antagonism toward virulent (disease-associated) *S. suis* strains. After the inoculation of the probiotic candidates, piglets were challenged with *S. suis* P1/7 (serotype 2) to test the ability of the selected strains to colonise newborn piglets; promote the development of a healthy microbiota; and prevent *S. suis* invasive disease after intranasal challenge with pathogenic strain P1/7. The candidate probiotic intervention did not lead to significantly different microbiota composition compared to the control group nor to significant reduction in mortality. However, differences were observed in the microbiota after *S. suis* challenge. One combination of candidate probiotic isolates was associated with higher piglet survival after *S. suis* challenge. And, we also detected relative high abundance and qPCR counts of *S. suis* in tonsillar microbiota compared to nasal cavity microbiota, which emphasises the tonsil as portal of entry in cases of invasive disease.

Chapter VI bring a discussion of the experimental results of the research and the novel insights inferred from these findings. Overall, the findings of this thesis support the notion that in future, key abundant taxa from tonsil microbiota in healthy piglets could contribute to prevention and control of post-weaning disorders. Finally, the research outcomes of this thesis have increased our understanding of *S. suis* pathobiology and niche colonization.

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The path leading to this point of finishing my PhD came with a lot of challenges and effort but also with so much fun and joy that I could never have imagined when I first decided to move abroad to pursue my PhD. This journey, or what we usually call "PhD life," not only taught me how to become a better researcher but also how to be a more compassionate and resilient person. But also important to note that all my personal and professional growth along those years wouldn't have been possible without all the beautiful people who, in one way or another, gave me their unique contribution, support, and love. And it is to all of you that I dedicate my following words.

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About the author

Isabela Maria Fernandes de Oliveira was born in Curvelo, Minas Gerais (MG), Brazil, on December 10, 1990. After successfully completing elementary and high school in her home-town, she decided to pursue BSc in Biological Science at the Federal University of Vicosa (UFV). Biology was always the subject that made her eyes shine brighter, but it was during her bachelor's studies that she discovered her interest in microbiology. She graduated with great success in 2015. In the second year of her studies, she started working as an intern at the Laboratory of Anaerobic Microbiology, Department of Microbiology (UFV). However, with the urge to always look for different experiences, Isabela was also involved in extracurricular activities such as the Biology Junior Enterprise and AIESEC. Those activities allowed her to work teaching in elementary schools, develop projects involving sustainability, being a team talent member receiving international volunteers, and organizing several events during the whole time of her studies.

Right after her graduation, she got a scholarship in the Post-Graduation program of the same university starting her Master's degree in Agricultural Microbiology with Prof. Hilário Cuquetto Mantovani. The master's project explored genomic approaches as well as culture-dependent techniques to discover novel antimicrobials in the rumen ecosystem. In the last six months (2016–2017) of her master's degree, she embraced the most challenging yet exciting experience of her life so far, she traveled abroad for the first time. She moved to Wales (UK) for a professional/academic exchange with Prof. Sharon Huws at the University of Aberystwyth, Institute of Biological, Environmental, and Rural Sciences where she worked with comparative genomics and prediction of antimicrobial peptides. This experience opened her mind to a world full of opportunities. After distinctly completing her master's in 2017, she decided to leave Brazil to pursue her PhD.

In 2018, she was awarded the Marie-Curie Fellowship and became a PhD candidate in the Host Microbe Interatomic Group at Wageningen University, the findings of her project are described in this thesis. In 2020, she received the EMBO (European Molecular Biology Organization) travel grant and visited the University of Warwick (Coventry/UK) in the Wellington Research Group for academic training focused on nanopore sequencing and heterologous expression of genes encoding secondary metabolites. As of April 2023, Isabela started working as a Researcher at the Swammerdam Institute for Life Sciences (SILS) at the University of Amsterdam (UvA) in the Molecular Analysis Department (MAD).

Raka Choudhury

List of publication

Oliveira, I. M. F., Taverne A. T., Vrieling M., Baarlen, P. v, Wells, J.M.. Macrophages and Monocytes in the lympho-epithelial tissues of porcine palatine tonsil: do they play a role in systemic infection of piglets with S. suis? (*To be submitted*)

Oliveira, I. M. F., Fredriksen S., Fernandez-Gutierrez M., Harmsen, H. J.M., Boekhorst J., Baarlen, P. v, Wells, J.M. Identification of novel species and biosynthetic gene clusters producing antimicrobial molecules through culturomics of the porcine tonsil microbiota. (*Submitted*)

Oliveira, I. M. F., Duncan Y. K. Ng, Baarlen, P. v, Marc Steggerb, Paal Skytt Andersenb, Wells, J.M. (2022). Comparative genomics of Rothia species reveals novel biosynthetic gene cluster diversity and ecological adaptation to different eukaryotic hosts and host niches. Microbial Genomics. https://doi.org/10.1099/mgen.0.000854

Oliveira, I. M. F., Neila-Ibáñez C., Fredriksen S., Correa-Fiz F., Boekhorst J., Baarlen P. v, Aragon V., Wells, J.M. Microbiota-based interventions to provide colonisation resistance against Streptococcus suis in early life. (*To be submitted*)

Oliveira, I. M. F., Godoy-Santos, F., Oyama, L. B., Moreira, S. M., Dias, R. G., Huws, S. A., Creevey, C. J., & Mantovani, H. C. (2022). Whole-Genome Sequencing and Comparative Genomic Analysis of Antimicrobial Producing *Streptococcus lutetiensis* from the Rumen. Microorganisms, 10(3), [551]. https://doi.org/10.3390/microorganisms10030551

Fredriksen S., Neila-Ibáñez C., Hennig-Pauka I., Guan X., Dunkelberger J., **Oliveira, I. M. F**., Correa-Fiz F., Aragon V., Boekhorst J., van Baarlen P., Wells J. M. (2022) *Streptococcus suis* infection on European farms is associated with an altered tonsil microbiome and resistome. bioRxiv 2022.08.01.500980; https://doi.org/10.1101/2022.08.01.500980

de Sousa B.L.*, Azevedo A.C.*, **Oliveira I.M.F.**, Bento C.B.P., Santana M.F., Bazzolli D.M.S., Mantovani H.C. PCR screening reveals abundance of bovicin-like bacteriocins among ruminal *Streptococcus* spp. isolated from beef and dairy cattle. J Appl Microbiol. 2021 Oct;131(4):1695-1709. doi: 10.1111/jam.15069

Overview of Completed Training Activities

The Basic Package (2 ECTS)	
WIAS Introduction Day (mandatory)	2018
Course on philosophy of science and/or ethics (mandatory)	2019
Disciplinary Competences (12 ECTS)	
Summer School on Microbiome in Health and Disease (SEEON, Germany)	2018
Network training in methods and tools in drug discovery and project presentation	2018
Writing research proposal (WUR)	2019
Bioinformatics teaching (NAI, UCPH, Denmark)	2019
Network training in bioinformatics (Warwick University, UK)	2019
Bacterial genomes: from DNA to protein function using bioinformatics	2019
Introduction to R (WUR)	2019
Professional Competences (15 ECTS)	
Presenting with impact (WUR)	2018
Communication and introduction to project management (NAI, UCPH, Denmark)	2018
Scientific Writing (WUR)	2019
Media training, commercialization and EU project check (Warwick University)	2019
Training in Open Access and project presentations and workshops (Warwick University)	2019
Searching and Organizing Literature (WUR)	2019
WGS PhD Workshop Carousel (WUR)	2019
Entrepreneurship, patenting and bringing drugs to market (Warwick University)	2020
Training Grant writing (UCPH, Denmark)	2020
Secondment At University of Warwick and writing EMBO grant	2020
Grant writing and career development (UCPH, Denmark)	2021
Societal Relevance (1 ECT)	
Public speaking course and FAMElab 2021 short talk	2021

Presentation Skills (4ECTS)

WIAS Science Day (Oral presentation)	2019
CARTNET presentations (EU-project check) (Oral presentation)	2020
Microbiome Interactions in Health and Disease (Oral presentation)	2020
PiGs European Scientific Meeting (Oral presentation)	2022

Teaching competences (5 ECTS)

Assistance in practical courses at WUR (HMI20306, HMI50306, HMI 30306) and supervision of BSc and HBO students	2019/2022

Total: 38 ECTS (one ECTS credit equals a study load of 28 hours)

Colophon

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