Streptococcus suis infection on European farms is associated with an altered tonsil microbiome and resistome

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Abstract

Introduction

Streptoccocus suis is a Gram-positive opportunistic pathogen causing systemic disease in piglets around weaning age. Outbreaks of *S. suis* disease are controlled by metaphylactic use of antibiotics, leading to high levels of antimicrobial resistance in *S. suis* isolates. This is an issue for both animal and human health due to the zoonotic disease potential of *S. suis*. The mechanisms facilitating invasive disease are not known but may involve host and environmental factors. The palatine tonsils are considered a portal of entry for pathogenic strains to cause systemic disease. We hypothesised that tonsil colonization by pathogenic and commensal bacteria may impact on disease risk via colonization resistance and co-infections. We conducted a case-control study on 9 European farms, comparing the tonsil microbiome of piglets with *S. suis* systemic disease with asymptomatic controls. We also compared these to piglets on control farms and piglets reared naturally in a forest.

Results

We found a small but significant difference in the tonsil microbiota composition of case and control piglets. Case-control associations varied between amplicon sequence variants (ASVs) and metagenome assembled genomes (MAGs) within the same species. Variants of putatively commensal taxa including *Rothia nasimurium* were reduced in abundance in case piglets compared to asymptomatic controls. Case piglets had higher relative abundance of *Fusobacterium gastrosuis*, *Bacteroides heparinolyticus*, and uncultured *Prevotella* and *Alloprevotella* species. There was, however, no higher abundance of *S. suis* itself at the species-level or of clinical strain marker genes in case piglets. Piglets sampled prospectively weeks prior to developing clinical signs had reduced microbiota alpha diversity. Despite case-control pairs receiving equal antimicrobial treatment, case piglets had higher abundance of antimicrobial resistance genes (ARGs) conferring resistance to antimicrobial classes used to treat *S. suis*.

Conclusions

The tonsillar microbiota of *S. suis* case piglets had increased abundance of taxa not previously linked to *S. suis* disease. This coincided with increased ARG abundance in case piglets, possibly due to adaptation of the disease-associated microbiota to frequent antimicrobial treatment.

Introduction

Streptococcus suis is a Gram-positive bacterium colonizing the upper respiratory tract of pigs. It is one of the major bacterial causes of disease in pigs and a zoonotic pathogen causing sepsis and meningitis in humans [1–4]. Swine infections are prevented by metaphylactic use of antimicrobials. This has led to increased antimicrobial resistance (AMR) in *S. suis* isolates [5], with macrolide and tetracycline resistance genes *erm(B)* and *tet(O)* being the most common [6]. The spread of antimicrobial resistance genes (ARGs) in zoonotic *S. suis* and to other streptococci is of concern to veterinary and human medicine [7].

The palatine tonsils have been suggested as a main habitat for *S. suis* colonization and a putative site of entry into the host bloodstream [8–15]. A recent study identified differences in taxonomic composition of the tonsillar microbiota in piglets diagnosed with *S. suis* disease [16]. Microbiota associations with *S. suis* infectious disease are of great interest to understand co-infection dynamics and to identify probiotic candidate species providing colonization resistance. It is thought that co-infection by bacterial and viral pathogens part of the porcine respiratory disease complex (PRDC) may predispose to *S. suis* invasive disease [17–19].

Tonsillar colonization by different *S. suis* strains may also impact on invasive disease risk [13, 20]. While *S. suis* has been described as an opportunistic pathogen, different strains have varying virulent potential and can be grouped into commensal and pathogenic clades [21–23]. Strains from pathogenic *S. suis* clades have reduced genome sizes [22] and putatively reduced ability to persist as colonizers in competition with commensals. Strains from pathogenic clades can also be isolated from the tonsils of healthy pigs, but it is not known whether *S. suis* invasive disease is preceded by outgrowth of disease-associated strains.

This study was aimed at identifying the tonsillar microbiota associated with *S. suis* systemic disease in weaning age piglets. We utilized a case-control study design and next generation sequencing to characterise the composition of the tonsillar microbiota of piglets with *S. suis* systemic infection at 9 European farms and compare these to piglets from 4 farms without *S. suis* disease. We used 16S rRNA gene amplicon- and metagenomic shotgun sequencing to quantify the tonsillar microbiota and resistome and analysed clinical and non-clinical *S. suis* strains from the farms by whole genome sequencing. This study design allowed us to assess microbiome composition and predict functionality, as well as to assess the abundance of marker genes prevalent in clinical or non-clinical *S. suis* strains.

Results

Sequencing

We sampled 45 case-control pairs of piglets with *S. suis* systemic clinical signs and asymptomatic control piglets from the same pen. Case-control pairs were equivalent in age, genetic background, and antimicrobial treatment. Additional control piglets from case farms and from control farms without *S. suis* outbreaks were sampled to assess farm differences. We also sampled one US farm and piglets raised organically in a forest for reference (see supplementary file 1 for information about the farms). Amplicon sequencing of the full set of 295 samples yielded an average of 76757 reads per sample after processing with DADA2 [24]. Metagenomic DNA from all case-control pairs and a subset of piglets from control farms was shotgun sequenced. A total of 109 metagenomic samples yielded on average 7.5 million reads per sample after removal of host DNA and plant DNA from feed.

The *Streptococcus suis* disease-associated microbiota

Case and control piglets on outbreak farms had significantly different tonsillar microbiota composition, but the effect size was small ($R2 = 0.01$ and $p = 0.02$, PERMANOVA on Bray-Curtis dissimilarity comparing case and control piglets within outbreak farms). Analysis per country found varying results for Spain ($R2 = 0.030$, $p = 0.01$) and Germany ($R2 = 0.017$, $p = 0.93$), which had fewer samples. The largest difference in tonsil microbiota composition was found on the Dutch farm NL1 ($R2 = 0.26$, $p < 0.01$), but this might be due to all 3 case piglets from this farm being sampled shortly after death allowing opportunistic pathogens to bloom. We did, however, not see a general association between the microbiota and clinical sign severity or (future) death within the other farms. In general, there was no consistent difference in alpha diversity between case and control piglets (Figure S1).

We identified amplicon sequence variants (ASVs) significantly associated with piglet case-control status (Figure 1A). SIAMCAT [25] identified 3 ASVs with significantly ($p < 0.05$) higher abundance in case piglets: ASV 29 (1.8% vs 0.7% mean abundance, *Prevotella* sp.), ASV 3 (3.6% vs 2.2%, *Alloprevotella* sp.), and ASV 21 (3.6% vs 1.5%, *Fusobacterium gastrosuis*). These were all part of the core microbiota in the European farms (minimum 99% prevalence in control piglets). The case-associations were stronger in the Dutch and Spanish farms, where the outbreaks were larger and/or symptoms more severe than in the German farms. These 3 ASVs also had the strongest association with case piglets using Wilcoxon Rank Sum Test ($p < 0.005$), but no ASVs were significantly different when applying multiple

testing correction (FDR > 0.05). No genera were significantly different between case and control piglets (FDR > 0.05). Table S1 lists case-control associations for all ASVs and genera.

While the 3 case-associated ASVs had the strongest case-control associations, few other ASVs trended towards case-associations. A larger number of different ASVs had weaker, non-significant (FDR > 0.05), control-associations. Among core microbiota members, ASV 48 (0.38% vs 0.74%, *Clostridium disporicum*), ASV 36 (0.47% vs 0.91%, *Rothia nasimurium*), and ASV 6 (1.98% vs 2.62%, *S. suis*) had the strongest control associations. ASV 11 (0.27% vs 0.63%, *Actinobacillus minor*, not present in farms ES4 and NL1) and ASV 81 (0.22% vs 0.40%, *Terrisporobacter mayombei*) were the non-core ASVs with the strongest control-associations. In terms of total relative abundance, ASV 1 (3.5% vs 8.1%, *Moraxella porci*/*pluranimalium*) showed the largest difference between case and control piglets. ASV 1 had strong control associations in farm ES2 (0.9% vs 5.6%, being completely absent in several case piglets) and ES3 (15% vs 24%) but was absent from farm ES4 and NL1 and equally abundant in German case and control piglets.

Porcine respiratory disease complex (PRDC) and its range of bacteria thought to co-infect with *S. suis* is mainly associated with respiratory disease in older pigs, while this study investigates weaning age systemic disease. Still, PRDC-associated bacteria were of interest in possible co-infections. *Streptococcus suis* itself and *Glaesserella parasuis* were among the most abundant tonsil microbiota members. Relative abundance of *S. suis* ASVs trended to be higher in healthy controls, while *G. parasuis* ASVs had mixed associations. ASV 174 (100% identical to both *G. parasuis* and *Actinobacillus indolicus*) trended towards case-association (0.27% vs 0.16%). *Pasteurella multocida* was relatively prevalent (50%) but at low abundance (mean 0.06%), although two outlier case piglets had over 1% abundance. *Actinobacillus pleuropneumoniae* had 34% prevalence and 0.08% mean abundance but was most abundant in control piglets. *Bordetella bronchiseptica* had lower prevalence (17%) but higher abundance (0.10%) due to some control piglets having up to 12% abundance. *Mycoplasma hyopneumoniae* was virtually absent from the dataset, with only 6 reads from a single control piglet.

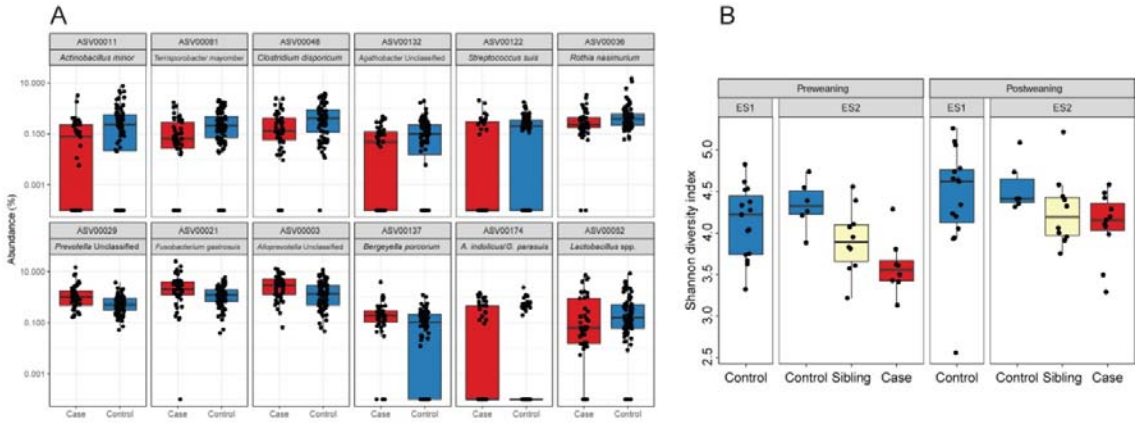

Figure 1: Microbiota case-control associations. **A.** ASVs significantly associated with case-control status on the outbreak farms, sorted by strongest case-control association with Wilcoxon Rank Sum Test. Top row: control-associated ASVs, bottom rom: case-associated ASVs. **B.** Shannon diversity (amplicon sequencing) of prospective samples collected pre-weaning from piglets that developed *S. suis* clinical signs 2-4 weeks later. Samples from future case piglets had lower Shannon diversity than control piglets which remained asymptomatic. Piglets from control farm ES1, which was managed by the same company and used sows from ES2, also had high mean diversity.

Early life microbiota diversity predicted clinical sign appearance weeks later

To assess the value of tonsillar microbiota composition to predict future invasive *S. suis* disease, we collected prospective samples on farm ES2, which had a history of post-weaning *S. suis* outbreaks, and farm ES1, a control farm without recorded *S. suis* problems despite use of sows produced on farm ES2. A cohort of 40 piglets were sampled on farm ES2 1 week before weaning, prior to visible clinical signs. Eight of the 40 piglets developed *S. suis* clinical signs 1-3 weeks post-weaning, and tonsil samples collected from these piglets post-weaning were included in the 45 case-control pairs.

Comparison of prospective samples collected from case and control piglets 1 week before weaning, i.e., 2-4 weeks before disease onset, revealed that the tonsil microbiota of case piglets had significantly lower Shannon diversity compared to control piglets that remained asymptomatic ($p = 0.005$; Figure 1B). Case piglets trended towards having lower diversity also post-weaning, during the outbreak, but the effect was smaller and less significant ($p = 0.12$). Asymptomatic siblings of case piglets also had a lower diversity than control piglets from litters without case piglets and piglets from control farm ES1 (Figure 1B). The difference in composition was, however, smaller 1 week pre-weaning ($R2 = 0.06$, $p = 0.15$,

PERMANOVA on Bray-Curtis dissimilarity) than during the outbreak ($R2 = 0.13$, $p < 0.01$). The Spearman correlation between ASV ratio of case-control abundance pre- and post-weaning on the farm was low ($R = 0.09$, $p = 0.4$), showing that separate ASVs were differentially abundant and driving case-control compositional differences pre- and post-weaning. The strongest pre-weaning case-associations were ASV 88 (*Pasteurellaceae*, 1.4% vs 0.55%) and ASV 186 (*Actinobacillus indolicus*/*minor*, 1.1% vs 0.21%), while ASV 77 (Leptotrichia, 0.11% vs 0.33%) and ASV 22 (Actinobacillus minor, 0.06% vs 0.20%) had the strongest control associations.

Metagenome-assembled genomes (MAG) analysis

We shotgun sequenced all case-control pairs and a subset of piglets from control farms. Co-assembly per farm produced 802 metagenome-assembled genomes (MAGs) (>70% completeness and <10% contamination). Read mapping to the MAGs yielded an overall case-control compositional difference similar to that found by amplicon sequencing analysis (Figure 2, $R = 0.03$, $p < 0.01$, Bray-Curtis dissimilarity PERMANOVA). The case-control associated MAGs largely corresponded to the ASVs identified by amplicon-based analysis, but some taxa, including case-associated *Bacteroides heparinolyticus*, were identified only using MAGs. The MAG approach resulted in stronger case-control associations (FDR < 0.0001) than the ASV approach, suggesting that the case-associated ASVs represented several strains with variable case-control associations. The two ASVs with strongest disease-association putatively corresponded to a collection of different *Bacteroidales* MAGs. The MAGs with the strongest disease-associations were classified as *Bacteroidales* UBA1309 and *Alloprevotella* F0040, clades poorly described and with few or no publicly available related isolate genomes. Case-control association of all MAGs are listed in Table S2.

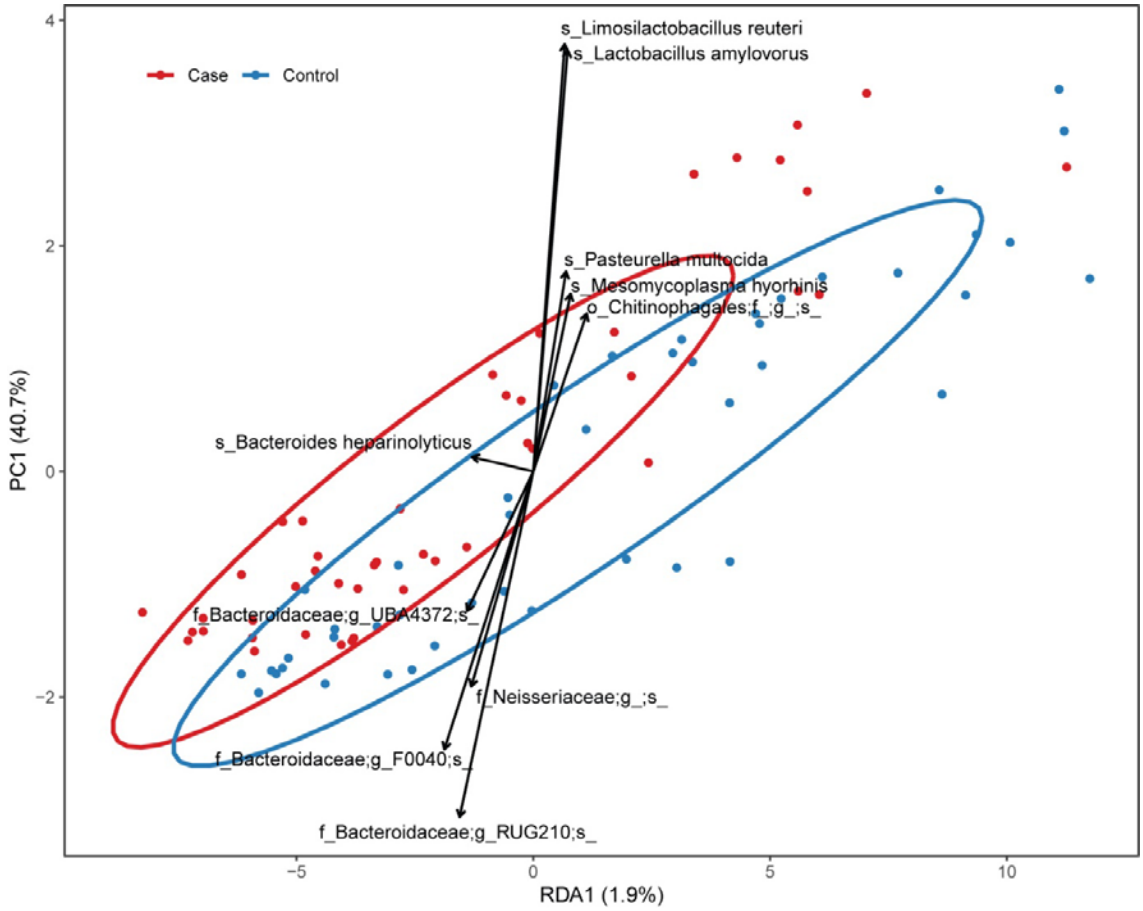

Figure 2: Redundancy analysis (RDA) on the abundance of metagenome-assembled genomes (MAGs) constrained by case-control status. The points represent individual samples; samples in the direction of arrows have more of that taxon. The eclipse represents 75% confidence level.

The tonsillar microbiome of piglets is similar between farms but diverged in free-range forest piglets

The tonsillar microbiota composition was more similar between piglets on the same farm than to that of piglets from other farms (Figure 3A-B). Compared to tonsillar microbiota compositions of farm piglets, Tamworth free-range piglets living outdoors in a forest in The Netherlands had a strikingly different microbiota and resistome (figure 3C-D). While European farm piglets shared a large core microbiota with 83 ASVs being present in 80% of piglets or more, 44 of these were not found in the free-range piglets. Vice versa of 157 ASVs found in all 5 forest piglets, 117 were not found in any farm piglet. Free-range piglets had low abundance of the genera most abundant in farm piglets, in particular *Moraxella* (0.6% vs 13%) and *Streptococcus* (0.9% vs 12%), and higher abundance of Acinetobacter (10% vs

2.3%), "*Rikenellaceae* RC9 gut group" (9.9% vs 0.1%), and *Treponema pedis* (3.6% vs 0.04%). The sample with the lowest *S. suis* abundance in the study, 0.3% abundance of a single ASV (ASV 1050), was from a free-range piglet. This ASV did not have 100% identity to the 16S rRNA gene V3-V4 region of any *S. suis* strain publicly available in SILVA or NCBI assembly databases. The other free-range piglets were colonised by *S. suis* ASVs shared with farm piglets.

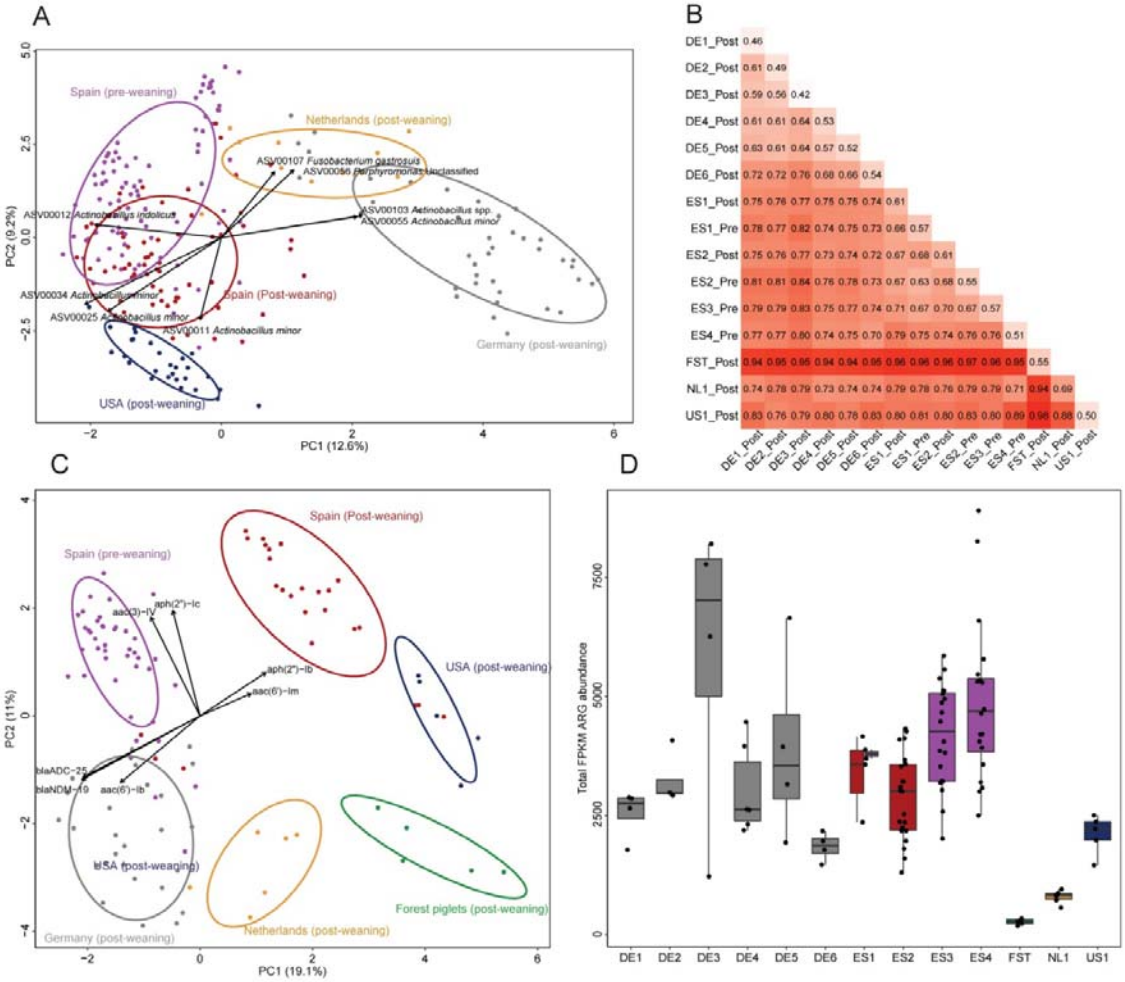

Figure 3: Microbiome differences between farms. **A.** PCA on microbiota composition (ASV abundance) of all farm samples and countries, pre- and post-weaning (transformed with log(1000*abundance+1)). Samples clustered broadly by country, but samples from farm DE6 clustered with NL1, and samples from ES4 clustered away from the other Spanish farms. **B.** Mean pairwise Bray-Curtis dissimilarity between samples from the different farms. **C.** PCA on ARG abundance (transformed with log(1000*abundance+1)). The pre- and post-weaning Spanish samples separated in 2 clusters. **D.** Total abundance of all ARGs per farm.

Increased tetracycline ARG abundance in case piglets

We quantified the abundance of antibiotic resistance genes (ARGs), collectively called the resistome, by mapping metagenomic reads to the Resfinder database [26] and normalizing abundance by fragments per kilobase reference per million fragments (FPKM). Piglets on most farms received antibiotics via feed or water, and these were included for resistome analysis. Twenty-two piglets received intramuscular injections, and these were excluded from the main analysis. All case-control pairs included in the main analysis had received equal antimicrobial treatment. Farms varied in total ARG abundance (Figure 3D), but shared high abundances of the most abundant ARGs, such as *blaROB-1* conferring resistance to penicillin/amoxicillin/ampicillin (411 FPKM mean abundance), *sul2* conferring sulfamethoxazole resistance (338 FPKM), and streptomycin resistance genes *aph(3'')-Ib* (312 FPKM) and *aph(6)-Id* (220 FPKM). Table S3 lists all detected ARGs per farm. The ARGs most common in *S. suis*, *erm(B)* and *tet(O)* [6], were less abundant (66 and 111 FPKM, respectively). When comparing the overall ARG composition by PCA, samples clustered by country, except for Spanish pre- and post-weaning samples clustering separately by age and not by farm (Figure 3C).

ARG abundance may, in addition to by taxonomic composition, be influenced by both historical antimicrobial usage on the farm and by direct antimicrobial treatment of the sampled piglets. This study could not disentangle these two effects since most antimicrobial treatments were given equally to all sampled piglets at each farm. Farm NL1, a high health status research farm where the sampled piglets were not treated, and where piglets are rarely treated with antimicrobials, had the lowest ARG abundance (except for the free-range forest piglets). In Germany, high health status farms DE1 and DE2 had low ARG abundance, but so did DE6 despite a history of severe *S. suis* disease. Farms DE3, ES3, and ES4 were assessed to have the lowest health status and highest historical antimicrobial usage by veterinarians (supplementary file 1), and piglets on these farms were also administered antimicrobials before sampling. These three farms had the highest ARG abundance (Figure 3D). There was no consistent link between the antimicrobials administered and the abundance of ARGs conferring resistance to these. On farm DE1, piglets that had received tetracycline had lower tetracycline ARG abundance than untreated piglets (810 vs 391 FPKM), and on DE3, where all piglets had received tetracycline, tetracycline ARG abundance was lower than on DE2 and DE5 where piglets had received no antimicrobial treatment (804 vs 805 and 926 FPKM, respectively).

The total abundance of ARGs was 15% higher in case piglets compared to controls (3473 vs 3026 mean FPKM), but this overall effect was not statistically significant (p = 0.36). ARGs conferring resistance to tetracycline (class level) were strongly case-associated (788 vs 620 FPKM, p = 0.01), especially within farms ES2 and ES3. Specifically, tetracycline and doxycycline resistance, conferred largely by the same ARGs, were the most abundant Resfinder ARG phenotype categories and had the strongest case-associations (Figure 4). *Tet(Q)* was the individual gene with the strongest case-association (134 vs 75 FPKM, FDR < 0.01). ARGs of classes aminoglycoside, beta-lactam, and macrolide, which like tetracyclines are commonly used to treat *S. suis* disease, also trended towards higher abundance in case piglets (Table S4).

We investigated the presence of case-associated ARGs in MAGs to determine if the higher ARG abundance was linked to the case-associated taxa. *Tet(Q)* was only found in case-associated *Prevotella* MAGs, and case-associated ARGs *ant(6)-Ib* and *tet(44)* were only found in case-associated *Fusobacteriales* MAGs. ARGs found in control-associated *Rothia* and *Clostridium* MAGs, *lnu(P)*, *erm(Q)*, *tetA(P)*, and *erm(X)*, were not case-associated.

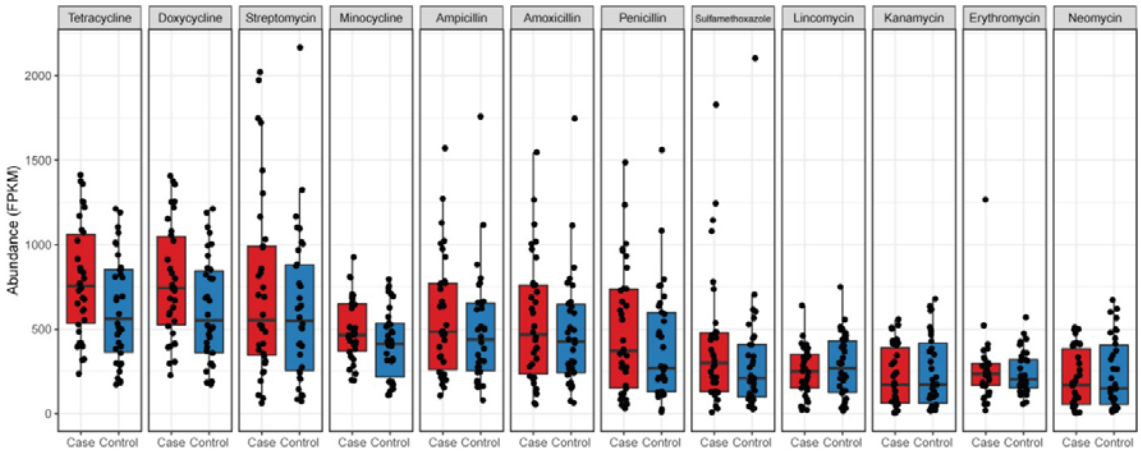

Figure 4: The abundance of 12 most abundant Resfinder ARG phenotype categories in case and control piglets. Note that most genes confer resistance to several antimicrobials, making the sum of phenotype abundances greater than the total ARG abundance. For instance, tetracycline and doxycycline resistance is conferred by the same genes.

Comparison of the tonsil and nasal microbiota

To evaluate whether differences in microbiota composition between case and control piglets were specific to the tonsillar microbiota or a general trend in the oropharyngeal cavity, we

collected 41 nasal swabs from three Spanish farms, ES1-3. Nasal and tonsillar swab microbiota composition was significantly different, but more variation was explained by the farm (R2 = 0.08, p < 0.001, Bray-Curtis PERMANOVA) than nasal/tonsil sample location (R2 = 0.06, p < 0.001). The nasal microbiota was characterised by higher abundance of genera *Moraxella* (32% vs 16%) and *Bergeyella* (5% vs 1%) and lower abundance of most other genera. Case and control piglets had a significantly different nasal microbiota (R2 = 0.07, p = 0.04, Bray-Curtis PERMANOVA). This effect size was comparable to the difference found in the tonsillar microbiota samples for the same subset of piglets (R2 = 0.07, p = 0.09). However, the ASVs that were differentially abundant in the nasal microbiota of case and control piglets were not the same ASVs that were differentially present in tonsillar microbiota. Some ASVs had inverse case-control associations: ASV 21 (*F. gastrosuis*), ASV 29 (*Prevotella* sp.), and ASV 3 (*Alloprevotella* sp.) were disease-associated in the tonsillar microbiota, both overall and within ES2 and ES3, but health-associated in the nasal microbiota.

S. suis **diversity in the tonsillar microbiota**

Amplicon sequencing data showed higher *S. suis* abundance in control piglets compared to case piglets on farms with pre-weaning outbreaks (farms ES3 and ES4, 3.9% vs 6.4%, p = 0.02). This effect was not significant on post-weaning outbreak farms (DE1, DE3-6, ES2, NL1, 4.4% vs 5.1%, p = 0.27).

In total 89 ASVs were classified as *S. suis*, and 52 of these were present in 2 or more tonsil samples, suggesting they are unlikely to be sequencing artefacts. Farm piglets were on average colonised by 7 *S. suis* ASVs, with a range between 2 and 16. *Streptococcus suis* relative abundance was similar in outbreak and control farms (Figure 5A). Comparison of the 89 *S. suis* ASVs to the 16S rRNA gene V3-V4 region of 2463 *S. suis* assemblies available on NCBI assembly revealed that many ASVs were poorly or not at all represented by sequenced genomes. This likely relates to the fact that many clinical but few non-clinical strains have been sequenced. ASVs are, however, not good markers for assessing *S. suis* strain diversity as the 16S rRNA gene V3-V4 region correlates poorly with whole genome phylogeny, and as both clinical and non-clinical strains share the same ASVs. 82% of all *S. suis* assemblies had a 16S rRNA gene V3-V4 region amplicon identical to ASV 6, which comprised 30% of *S. suis* in the tonsillar microbiota. ASV 17, the second most abundant *S. suis* ASV, comprised 21% of all *S. suis* in the microbiota but was only found in 0.08% of the assemblies. In

addition to strain DE512T1 collected in the present study, ASV 17 was only found in 2 isolates recently sampled from diseased pigs in China (GCF_019793915.1 and GCF_019794525.1).

Further, we used metagenomic data to assess the relative proportion of commensal and pathogenic *S. suis* in the tonsillar microbiota of each piglet. We created a *S. suis* pangenome by clustering protein coding genes from a previously published genome collection [21] at 80% identity, and mapped metagenomic reads to the representative sequences of each cluster to assess their abundance in each sample. We calculated the ratio of prevalence of each cluster in clinical and non-clinical genomes to assess their putative association with pathogenicity. We found that in the tonsillar microbiota, genes predominantly found in non-clinical strains occurred at higher abundance than genes common in clinical isolates (figure 5B). There was a small positive correlation between gene clinical/non-clinical genome presence ratio and case-/control sample abundance ratio (Figure 5C, Spearman $R = 0.05$, $p < 0.01$), due to higher abundance of commensal *S. suis* in control samples.

One often used *S. suis* marker gene for strain virulence is the gene encoding suilysin (*sly*), and this gene was found in at least 1 clinical strain from all farms where we sequenced clinical strains. *Sly* is well suited for use in metagenomics analysis due to high sequence conservation across *S. suis* strains. *Sly* abundance was low in the tonsillar metagenomes, and similarly abundant in case and control piglets (Figure 5D). On some farms we did not detect *sly* in any piglets, but this may be due to small sample size and insufficient sequencing depth.

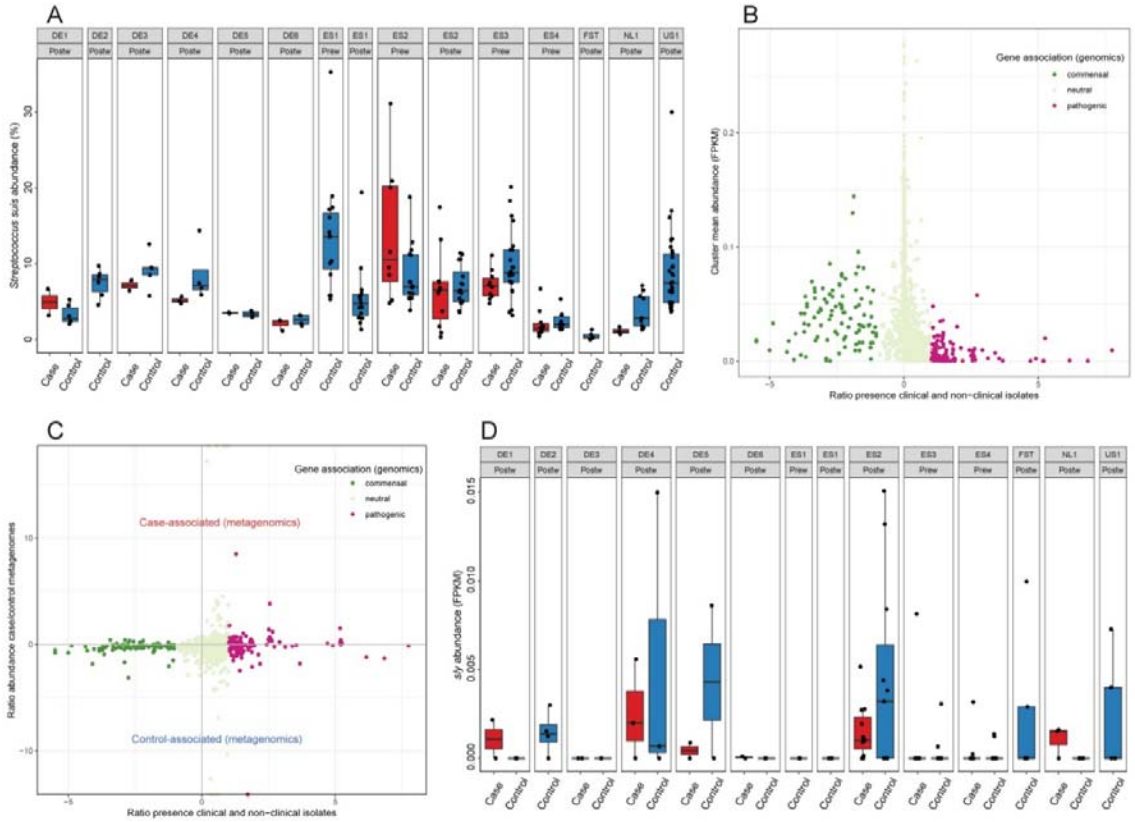

Figure 5: *S. suis* abundance and diversity. **A)** Total *S. suis* relative abundance per farm, amplicon sequencing data. **B)** Within the *S. suis* pangenome, genes associated with non-clinical strains (x-axis) were more abundant (y-axis) in metagenomic samples from the tonsillar microbiota. Genes with ratio 0 are equally common in clinical and non-clinical strains, genes with a ratio of 1 are twice as common in clinical strains. **C)** The correlation between gene association with clinical/non-clinical genomes (x-axis) and association with abundance in case-control metagenomic samples. **D)** The abundance of suilysin encoding gene *sly*, a gene highly conserved in clinical strains, in case and control samples per farm.

Discussion

In this study, we found the tonsil microbiota composition of case piglets with *S. suis* clinical signs to differ significantly from the microbiota of asymptomatic controls. *Streptococcus suis* disease may occur as a part of polymicrobial infections collectively known as porcine respiratory disease complex (PRDC), which includes porcine reproductive and respiratory syndrome (PRRS) virus, swine influenza A and potentially bacterial primary and secondary (opportunistic) pathogens [18, 27]. We did not find disease-associations with the tonsil microbiota abundance of any species linked to PRDC, such as *G. parasuis* or *S. suis* itself.

This study included only piglets with weaning age systemic *S. suis* disease, and PRDC associated taxa may be more relevant to respiratory disease in older finisher pigs.

We identified novel disease-associations with *Fusobacterium gastrosuis*, *Bacteroides heparinolyticus*, and uncultured *Prevotella* and *Alloprevotella* species. *Fusobacterium gastrosuis* has previously been linked to *Helicobacter suis* infection in the gastric microbiota and shown to have genes involved in adhesion, invasion and induction of cell death as well as in immune evasion in other *Fusobacterium* species [28]. The uncultured case-associated *Alloprevotella*/*Prevotella*/*Bacteroides* species lack isolate genomes and are unknown in relation to *S. suis* disease, possibly due to being unculturable. Case-associated taxa may also interact with the host to facilitate *S. suis* to cross epithelial barriers without themselves entering the bloodstream, thus remaining undetected by necropsy. Alternatively, disease-associated taxa may increase in abundance due to host immune status and dysbiosis, as suggested for oral *Prevotella*/*Alloprevotella* species in humans [29].

Case piglets had lower tonsillar *S. suis* abundance than control piglets. We assessed that this was due to a reduced abundance of strains from commensal clades, most of which are poorly represented among sequenced *S. suis* strains. While *S. suis* genes predominantly found in non-clinical isolates were more abundant in control piglets, genes predominantly found in clinical strains, such as *sly*, were low in abundance and more equally distributed between cases and controls. This shows that the majority of *S. suis* colonising tonsils are commensal, lacking genes required for invading the host, but also confirms that strains carrying genes most prevalent in clinical strains are also colonising asymptomatic piglets at low abundance. Based on these results, we conclude that tonsillar colonisation by *S. suis* itself cannot be used to reliably predict or even confirm ongoing *S. suis* invasive disease.

Associations between *S. suis* disease and the tonsillar microbiota have been investigated using amplicon sequencing in a previous study by Niazy et al. [16]. *Bacteroides* and *Lachnospiraceae* were found to be more abundant in controls, while case piglets had higher abundance of *Campylobacter* and *Porphyromonas*, among others. This may be due to European and North American piglets having fundamentally different microbiomes. Differences between studies may largely be due to methodological differences, but in the present study we included one US farm and found the sampled piglets to have low diversity, and that while microbiota members were shared at the ASV level, composition was dominated by high *Actinobacillus* and *Streptococcus* abundance. The different findings in

Niazy et al. might also be due to their sampling of whole tonsillar tissue while we used swabs. Furthermore, piglets suffering from other pathologies such as rectal prolapse and hernia were sampled as controls, so the case piglets were not compared with healthy controls as in the present study. While their study may include control-associated taxa associated with other disease, our results may not be specific only to *S. suis* disease but include microbiome traits associated with low health status in general. Niazy et al. also sampled uneven numbers of case and control piglets per farm, and from some farms no controls. This confoundment of case-control and farm comparison may have affected the results.

We found case piglets to have higher abundance of antimicrobial resistance genes than controls, despite case-control pairs being treated with the same antimicrobials. ARGs conferring resistance to doxycycline and tetracycline had the strongest case-association, and ARGs conferring resistance to other antimicrobials used against *S. suis* also trended towards being more abundant in case piglets. ARGs found in MAGs of disease-associated taxa *Fusobacterium*, *Prevotella*, and *Alloprevotella* had strong disease-associations compared to those found in health-associated *Rothia* and *Clostridium* MAGs. It is possible that frequent antimicrobial usage has caused selection pressure on disease-associated taxa, leading to accumulation of ARGs.

While antimicrobial treatment may have caused strong selective pressure over time, we found limited increases in abundance of ARGs conferring resistance to the antimicrobials used to treat the sampled piglets. It was unexpected to find that antimicrobials administered by water and feed did not appear to have led to increases in ARG abundance. Antimicrobial usage starting in the first few days after birth has previously been shown to influence piglet nasal microbiota composition and diversity [30], but in the present study most antimicrobial treatment started later in life, and only a few days before sampling. Another plausible explanation for the limited effect of antimicrobial treatment on the tonsillar microbiota in our study is intrinsic resistance of biofilms to antibiotics [31]. The route of antimicrobial administration is known to determine the impact on gut microbiota [32, 33], but it is not known to what extent antimicrobials provided in water, feed, or by injection are able to penetrate oral biofilm.

Farms had significant differences in microbiota composition, but clustered by country. This may be due to both sampler bias (a single person collected all samples in each country) and farm environment, practices, and regulations that vary by country. The piglet microbiota may

for instance be influenced by factors such as cleaning, feed composition, temperature, and antimicrobial treatment [34–41]. However, the microbiota composition was not more similar on farms with comparable management practices. The genetics and source of the sows, and their vertically transferred microbiota may be a more important determinant of the piglet microbiota than farm conditions. Among the Spanish farms, ES1 and ES2, which were operated by the same company and had frequent exchange of animals, had the most similar microbiota composition to each other. However, unrelated German farms shared a more similar microbiota composition than any of the Spanish farms. In both Spain and Germany, farms were spread over a large geographic area and managed by different companies.

We sampled the tonsillar microbiota of 5 piglets living outdoors in a forest in The Netherlands. These piglets have no recorded problems with diseases commonly affecting pigs on intensive farms, including *S. suis* associated disease. We found the tonsillar microbiota of the forest piglets to be fundamentally different from farm piglets, and to have lower ARG abundance. Core ASVs in farm piglets were completely absent in the free-range piglets, and core ASVs from the forest piglets were not found in farm piglets. The ecological farm DE4, where piglets had straw bedding and outside access, did not have a more similar microbiota to the forest piglets than other farms. Compared to DE4 and other farms, living outdoors may shape the tonsillar microbiota via exposure to environmental microbes from soil and diverse natural feed sources, and via high air quality and lower exposure to bacterial transfer from other piglets. Previous studies have shown differences in the faecal [42–44] and nasal [45] microbiota of wild pigs, but we are not aware of studies on specific factors that may drive differences between the natural and domestic microbiota or between farms with varying disease problems. Understanding these factors may be key to preventing disease by opportunistic pathogens in pig farming.

We conducted a longitudinal sampling on farm ES2, collecting prospective samples from pre-weaning, prior to clinical sign development. Future case piglets had reduced alpha diversity compared to asymptomatic controls and piglets from control farm ES1. Asymptomatic siblings of case piglets had alpha diversity intermediate between symptomatic siblings and control piglets from litters without any *S. suis* cases. *Streptococcus suis* disease cases were concentrated in a limited number of litters, despite most of the herd remaining unaffected. This suggests that maternal effects involving early life immunity- or microbiota may be important in predisposing to *S. suis* disease and potential dysbiosis. This may be due to vertical transmission of a disease-prone microbiota, but also differences in maternal

immunity, with antibodies depleting prematurely [46]. *Streptococcus suis* disease most commonly occurs around the time colostral antibodies to *S. suis* start becoming depleted. Various studies have found other colostral antibodies to have similar half-lives as well as high variation in antibody abundance and half-life between individual piglets and litters of different sows [47–51]. Thus, it is possible that during *S. suis* outbreaks, some piglets have sufficiently high levels of maternal antibodies to opsonise invading *S. suis*, whereas other piglets in some litters lack a sufficient level of maternal immunity against *S. suis*.

In conclusion, there are small but significant differences between the tonsillar microbiota of *S. suis* case piglets and asymptomatic controls. We discovered novel taxa associated with case piglets, while *S. suis* abundance was higher in controls. The microbiota differences may originate from dysbiosis starting during early life prior to disease outbreak, but further research is needed to assert this. It is also not conclusively known whether *S. suis* invades through the tonsils or other locations. Case piglets had higher abundance of ARGs conferring resistance against classes commonly used to treat *S. suis* disease. This may be linked to high ARG prevalence in case-associated taxa driven by more frequent exposure antimicrobial treatment than control-associated taxa.

Materials and methods

Animals

We utilized a case-control study design to assess the association between tonsillar microbiota composition and incidence of *S. suis* invasive disease. To search for consistent correlations between microbiota composition and *S. suis* disease incidence we included farms from different countries with different livestock management systems (supplementary text 1). Tonsil swabs were obtained from 3- to 10-week-old piglets at 13 farms, of which 9 had ongoing *S. suis* disease outbreaks. Three sampled farms had no history of *S. suis* disease. We compared our European farms to samples obtained at one US farm with a history of *S. suis* disease but no cases at the time of sampling. Lastly, we sampled 5 piglets free-living in a forest in the Netherlands at approximately 1 month after separation from the sows.

We selected 45 pairs of case-control piglets for metagenomic sequencing (table 1). The pairs were selected to be as equal as possible, coming from the same pen, room, and/or sow, and having received equivalent antimicrobial treatment. The cross-sectional case-control study design was extended with longitudinal sampling at two farms, ES1 and ES2, to determine whether microbiota differences in early life may predict future *S. suis* disease occurrence.

ES1 is a production farm free of *S. suis* disease despite all sows and their carried microbiota and *S. suis* strains originating from ES2, which had severe *S. suis* disease problems.

Clinical signs consistent with *S. suis* infection were recorded at each farm visit. Cases fell into two categories: arthritis (typically presenting as lameness) and meningitis (including otitis and sepsis, typically presenting as loss of balance, paralysis, paddling, shaking, and convulsing). Confirmation of *S. suis* infection was not carried out on the individual piglets due to welfare reasons. Most sampled piglets recovered after antimicrobial treatment. *Streptococcus suis* disease was confirmed by necropsy in all 3 case piglets on farm NL1.

This study and all animal procedures were approved by the appropriate ethical committees. Sampling of the forest piglets was conducted according to the restrictions of the Animal and Human Welfare Codes in The Netherlands (2019.W-0026.001). On other farms, sampling was carried out for diagnostic purposes (covered by EU Directive 2010/63/EU).

Table 1: Number of case and control samples collected from each farm. See detailed information in supplementary text 1.

			Amplicon		Metagenomics	
Farm	**Country**	**Age**	**Case**	**Control**	**Case**	**Control**
DE1	Germany	Postweaning	2	6	2	2
DE2	Germany	Postweaning		6		4
DE3	Germany	Postweaning	2	5	2	2
DE4	Germany	Postweaning	3	4	3	3
DE5	Germany	Postweaning	2	4	2	2
DE6	Germany	Postweaning	3	4	2	2
ES1	Spain	Postweaning		15		3
ES1	Spain	Preweaning		15		2
ES2	Spain	Postweaning	11	16	11	11
ES2	Spain	Preweaning	8 prospective	16		
ES3	Spain	Preweaning	10	22	10	10
ES4	Spain	Preweaning	10	10	10	10
FST	Forest	Postweaning		5		5
NL1	Netherlands	Postweaning	3	9	3	3
US1	USA	Postweaning		30		5

Whole genome sequencing of bacterial isolates

We selected 9 clinical (isolated from lesions observed at necropsy on the farms, but not necessarily from the same piglets sampled for tonsillar microbiota) and 7 non-clinical (from tonsillar swabs) *S. suis* strains for whole-genome sequencing. The isolates were grown in Todd-Hewitt broth with yeast extract overnight, and DNA was isolated with PowerSoil DNA

Isolation Kit (Qiagen) with 0.1mm silica bead beating. Isolated DNA was paired-end Illumina sequenced. Reads were trimmed with trimmomatic v0.39 [52], assembled with Spades v3.14.1 [53], and annotated with Prokka v1.14.5 [54]. Strain metadata and assembly statistics is shown in Table S5.

Sample collection

The palatine tonsil microbiota of piglets was sampled by gently scraping the tonsillar surface with HydraFlock swabs (Puritan, ME, USA) for 10 seconds. Swabs were immediately put in vials containing Powerbead solution (Qiagen, The Netherlands) and transported at -20 □C before being stored at -80 □C. DNA was isolated using the PowerSoil DNA Isolation Kit with 0.7mm garnet bead beating according to the manufacturer's recommended protocol.

Amplicon sequencing

The V3-V4 region of the 16S rRNA gene was amplified with primers 341F (5′-CCTAYGGGRBGCASCAG-3′) and 806R (5′-GGACTACNNGGGTATCTAAT-3′) and paired-end 250 bp sequenced using either Illumina HiSeq 2500 or Novaseq 6000. Reads were trimmed with cutadapt 2.3 [55] using default settings before being processed in DADA2 [24] following the v1.4 workflow for paired-end big data. Different sequencing batches were run separately before being merged to account for differences in the learnErrors step. Taxonomy was assigned with SILVA database v138 [56]. Genus level taxonomy was assigned by the DADA2 pipeline using the RDP Naive Bayesian Classifier algorithm, and we used mmseqs2 easy-search with default settings to detect the species in SILVA with the highest identity alignment to each ASV [57]. The highest identity species alignment above 98.5% was assigned. When several species had equally high identity all were assigned separated by a slash. Amplicon sequence variants (ASVs) with taxonomic assignment as eukaryote, mitochondria, or chloroplast were discarded. Alpha and beta diversity were calculated on rarefied data (24325 reads) using R packages Phyloseq [58] and vegan [59], and the vegan::adonis function was used to perform PERMANOVA to determine the overall compositional differences between groups. Vegan function RDA was used for principal component analysis (PCA) and redundancy analysis (RDA).

Shotgun sequencing

Metagenomic libraries were prepared with NEB Next Ultra DNA Library Prep Kit (New England Biolabs, ME, USA) following the manufacturer's instructions. DNA was fragmented

to 350 bp, purified with AMPure XP (Beckman Coulter, CA, USA) and sequenced with 150 bp paired-end sequencing on an Illumina NovaSeq 6000 machine.

Analysis of metagenomic data

Pig and plant (feed) reads were removed with kneaddata (https://github.com/biobakery/kneaddata) using the genomes of pig (GCF_000003025.6), wheat (GCF_002162155.1), and maize (GCF_902167145.1). Since some samples still had a large proportion on host or plant reads left after this, we further normalized read counts by the proportion of plant and pig reads found by kraken analysis [60]. This prevented samples with small proportions of bacterial reads from being outliers when mapping metagenomic reads to marker genes and metagenome assembled genomes (MAGs). We created MAGs using MetaWRAP v1.3.2 [61] with SPAdes v3.14.1 [53], and identified MAG taxonomy with GTDBtk v1.3.0 [62] and ARGs with resfinder software v4.1 [26]. We also used the resfinder database for quantifying ARG abundance directly from metagenomics reads. Before mapping we clustered the genes in the database to 90% identity using mmseqs2 [57] easy-cluster with settings "--min-seq-id 0.9 --cov-mode 0". Reads were mapped to the representative sequences of the clusters using mmseqs2 easy-search with default settings, and reads aligning with minimum 100 bp and at 95% identity were accepted.

To assess the *S. suis* population in the tonsillar microbiota we created a *S. suis* pangenome by clustering all protein coding genes and mapping metagenomic reads to representative sequences. We annotated the genomes using Prokka v1.14.5 and clustered all protein coding genes at 80% residue identity using mmseqs easy-cluster (–min-seq-id 0.8 –cluster-mode 2 –cov-mode 1). We used previously published metadata covering 1703 assemblies [21]. We determined the association of each cluster with presence in clinical and non-clinical strains by calculating the ratio of percent presence in each group. The ratio of clusters more present in clinical strains were calculated by (% presence clinical/% presence non-clinical) - 1, and clusters more present in non-clinical strains by ((% presence non-clinical/% presence clinical) * -1) + 1, so that clusters equally prevalent in clinical and non-clinical strains had a ratio of 0. We mapped metagenomic reads to the representative sequence of each cluster as described above and accepted reads mapping at 80% identity and 80% length.

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Availability of data and materials

All microbiome sequencing data generated by this study is deposited in the NCBI BioProject portal under accession number PRJNA854341. Genome assemblies are available under accession numbers PRJNA849547 and PRJNA849577.

Authors' contributions

VA, FCF, PB, and JW conceived the study. SF, CNI, IHP, XG, JD, IFO, and MLF performed field sampling and sample processing. SF, FCF and JB performed bioinformatic and statistical analysis. All authors contributed to editing the manuscript and read and approved the final version.

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