RESEARCH REPORT
PORK SAFETY

Title: Evaluation of the nasal microbiome and its potential role in MRSA colonization in pigs – NPB #11-096

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Industry Summary:

Livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA) has emerged in the past 10 years as an important public health concern. Since first recognition in Europe, LA-MRSA has been identified in pigs in various countries on at least 4 continents, and significant concern has been raised about the potential impacts on pig farmers and veterinarians, rural residents and the broader population. Once on a farm, high rates of MRSA carriage can be encountered in pigs, but it is rarely an 'all-or-nothing' event, with MRSA-negative pigs being found on farms with high MRSA carriage rates. This raises questions about why some pigs are colonized while other remain MRSA negative. A variety of factors may be involved, with one possibility being differences in the nasal bacterial population (microbiota) and its overall genetic composition (microbiome). While not adequately studied, it has been assumed that the pig has a complex and abundant bacterial population in its nasal passages, and that may play a critical role in determining the fate of bacteria to which it becomes exposed. The objective of this study was to describe the nasal microbiome of slaughter-age pigs and to evaluate the influence of the microbiome on MRSA colonization.

Nasal swabs were collected from farm- and age-matched pigs. Swabs were screened for MRSA, and positive and negative pigs were selected for microbiome study using a 2nd swab that was collected. Using molecular methods, the nasal bacterial microbiome was described and the two groups were compared. To further evaluate the influence of management on the nasal microbiome, swabs were collected from a set of age-matched pigs on a farm that fed a liquid diet.

The nasal cavity of the pig contains a diverse and complex microbial population, with an average of close to 100 different bacterial species per pig. There was no significant difference in the number of different species, the bacterial diversity or the overall bacterial population composition in MRSA positive versus negative pigs, although there were significant differences in some bacterial groups.

Interestingly, there was a marked difference in the nasal microbiome between conventionally-fed and liquid-fed pigs. Liquid diet-fed pigs had a much different overall nasal microbial population, with significant differences at the Phylum level, consisting of greater proportions of Firmicutes and lesser proportions of Bacteroidetes and Proteobacteria.

This study has provided the most comprehensive understanding of the nasal microbiome of the pig, something that may be relevant for understanding of various bacterial (and perhaps viral) pathogens that can reside in the nasal cavity. It has shown that the nasal microbial population does not exert an apparent effect on the likelihood of MRSA shedding in pigs, but some minor population differences that were present deserve further study. The finding of a pronounced difference between pigs fed different diets indicates the potential for management practices to have a profound impact on the nasal microbiome, something that should be considered when studying a range of pig and zoonotic pathogens.

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Scientific Abstract: Since first recognition of livestock-associated MRSA in Europe, significant concern has been raised about the potential impacts on pig farmers and veterinarians, rural residents and the broader population. A better understanding of factors that affect MRSA carriage by pigs is needed to develop effective control programs. While not adequately studied, it has been assumed that the pig has a complex and abundant bacterial population in its nasal passages, and that may play a critical role in determining the fate of bacteria to which it becomes exposed. The objective of this study was to describe the nasal microbiome of slaughter-age pigs and to evaluate the influence of the microbiome on MRSA colonization.

The nasal microbiome of age- and farm-matched MRSA carriers and non-carriers was evaluated and compared. To further evaluate the influence of management on the nasal microbiome, swabs were collected from a set of age-matched pigs on a farm that fed a liquid diet.

The swine nasal microbiome is complex and highly variable between individuals. Species richness is high, with between 25 and 277 species identifications per animal (mean 120, median 98). There was no difference in richness between MRSA carriers and non-carriers (P=0.94).

Overall, the Proteobacteria Phylum was most abundant. Firmicutes was the second most common Phylum overall and was predominant in some individuals (largely based on high abundances of *Staphylococcus*, *Bacillus* and *Paenibacillus* spp). The following other phyla were identified in decreasing relative abundances: Bacteroidetes, Spirochaetes, Actinobacteria, Cyanobacteria, Thermotogae, Tenericutes, Synergistes, Fibrobacteres, Fusobacteria, Elusimicrobia, Lentisphaerae and Deferribacteres. The microbial population structure did not differ between groups (Parsimony test P>0.05). Using principal component analysis, there was no apparent clustering of the MRSA carriers. There were few differences between groups at lower taxonomic levels, with no differences at the Phylum or Class level.

While there was no difference between farm-matched MRSA positive and negative pigs, there was a significant difference between those pigs and pigs from a different farm that were liquid diet-fed. Liquid-fed pigs had significantly fewer Bacteroidetes and Proteobacteria, and more Firmicutes. There were numerous differences between groups at lower taxonomic levels. This indicates that management factors can influence the nasal microbiome, so further comparison of factors that modify the nasal microbiome and the corresponding influence on MRSA (as well as other swine or zoonotic pathogens) is indicated.

This has been the first comprehensive study of the porcine nasal microbiome and it has demonstrated a highly complex microbial population. There was no evidence that the endogenous nasal microbiota is a key factor in determining whether MRSA will be able to colonize an individual pig. Therefore, other measures to control this concerning bacterium are indicated. However, this study has provided the first insight into the normal composition of the nasal microbiome and identified that management factors (diet) can influence it. It is possible that differences in the nasal microbiome could influence various aspects, such as the immune response and carriage of various bacterial and viral swine and zoonotic pathogens. This study has provided critical baseline information about the composition of the nasal microbiome and future study of the role of this microbiome (as well as the microbiomes of other body sites) is indicated.

Introduction: Methicillin-resistant *Staphylococcus aureus* (MRSA) emerged in humans in the 1960s, and by the 1980's was a leading cause of hospital-associated infections. In the 1990s, community-associated MRSA

was identified in people, and certain MRSA strains have spread in epidemic fashion internationally, resulting in large numbers of infections and deaths. A dramatic change in the understanding and epidemiology of MRSA occurred in the 2000s with the emergence of livestock-associated MRSA (LA-MRSA). First recognized in pigs in the Netherlands, LA-MRSA has now been identified in pigs on at least 4 continents. While of little consequence to pig health, LA-MRSA is a significant public health concern, since LA-MRSA can also infect people. Infections were first noted (and remain most common in) pig farmers and veterinarians, but infections can occur more broadly, in family members of pig farmers, rural residents and the community-at-large. In some areas in Europe, LA-MRSA is a leading cause of community-associated MRSA infections in people. In the US, LA-MRSA infection in people appears to be uncommon, but there is concern that it may be an un- or under-recognized problem since human surveillance focuses on hospitals and outbreaks, as opposed to sporadic infections in the community. Regardless, LA-MRSA is widely distributed in the pig population in North America and high rates of MRSA carriage can be found in US pig farmers. On some farms, the majority of pigs can be carriers, and MRSA can be easily distributed between farms by breeding stock. While high rates of MRSA carriage can be encountered in pigs on a specific farm, it is rarely an 'all-or-nothing' event, with MRSA-negative pigs being found on farms with high MRSA prevalences. This raises questions about why some pigs are colonized while other remain MRSA negative. A variety of factors may be involved, with one possibility being differences in the nasal bacterial population (microbiota) and its overall genetic composition (microbiome). While not adequately studied, it has been assumed that the pig has a complex and abundant bacterial population in its nasal passages, and that may play a critical role in determining the fate of bacteria to which it becomes exposed. The objective of this study was to describe the nasal microbiome of slaughter-age pigs and to evaluate the influence of the microbiome on MRSA colonization.

Objectives:

1. To describe the composition of the nasal microbiome of slaughter-age pigs
    a. The nasal cavity presumably consists of a complex and diverse bacterial population and this population may play a critical role in preventing or eliminating colonization of bacteria like MRSA. Understanding the composition of this population is the first step in development of flora-modification approaches for MRSA colonization prevention or elimination.
2. To compare the nasal microbiome of slaughter-age pigs that are or are not colonized with MRSA.
    a. Determining differences in the nasal microbiome of colonized and non-colonized pigs may identify specific organisms or groups of organisms that play a role in preventing colonization. This could then be used for development of approaches to prevent or eliminate colonization.

VII. Materials & Methods

1. Farms that have previously been identified as having MRSA-colonized pigs were enrolled.
2. Nasal swabs were collected from 400 pigs within 1 week of slaughter. Double swabs were used.
3. Selective MRSA culture was performed on one swab using a standard enrichment broth followed by inoculation onto MRSA Chromogenic agar. MRSA was identified using standard methods.[1]
4. A final study population of swabs from farm matched MRSA positive and MRSA negative pigs was selected. The second swabs were subjected to DNA extraction using a robotic magnetic beads-based system. PCR amplicon libraries of the V5-V6 hypervariable region of the 16s rRNA gene were develop using an established primer set. MID tags were added to PCR products for emulsion PCR to allow for multiplexing.
5. Sequencing was performed in a 454 next generation sequencer (Roche GS Junior Sequencer).
6. The sequences generated by pyrosequencing were cleaned using the comprehensive bioinformatics software package, mothur (version 1.23.1) (Schloss et al., 2009). This software was used to trim barcode and primer sequences, and to remove homopolymers greater than 8bp, and sequences shorter than 200bp, allowing for 1 mismatch to the barcode and 2 mismatches to the primer.

Chimeras were identified with the "chimera.uchime" command, using the most abundant sequences as a reference. The refined data was uploaded into MG-RAST (Meyer et al., 2008) to taxonomically assign the individual reads to known DNA sequences and to observe inter-animal variation. Phylogenetic profiles were generated using the SILVA Small Subunit rRNA Database (SSU), with a maximum e-value of 30, a minimum percent identity of 97, and a minimum alignment length of 50. Analysis was restricted to the domain Bacteria.

7. Sequences were aligned to the SILVA-based bacterial reference alignment using the Needleman-Wunsch algorithm in mothur. Reads containing ambiguous bases, exclusively gap characters, or with regions strictly outside of the desired region of the 16S gene were removed, and sequencing noise was reduced from the dataset using the "pre.cluster" command. Sequences were assigned to operational taxonomic units (OTUs) based on a phylip-formatted lower triangle matrix, using the 'furthest neighbor algorithm' at 97% sequence similarity. Sequences from each OTU were taxonomically assigned with a bacterial 16S rRNA Silva reference alignment using a naïve Bayesian classifier. A consensus threshold of 80% was applied with a distance of 0.03. To acquire the consensus taxonomy of these OTUs, sequences were aligned to a 16S rRNA Silva reference alignment.
8. To normalize the number of sequences in the OTU dataset and to remove spurious OTUs, the data was subsampled to the minimum number of sequences in a sample (9325bp). OTUs were used to calculate community diversity (Shannon and Simpson diversity indices), evenness (Shannon equitability index) and richness (Chao1) to a cutoff of 0.03. Completeness of sampling effort was evaluated using Good's coverage and rarefaction curves to a cutoff of 0.03 for each sample. Dendrograms were created using MOTHUR to compare the similarity of the intestinal bacteria among all samples used in the study using both, the Jaccard index and the Yue & Clayton measure, which account for the relative abundances in each sample. Figures were generated by TreeView 1.6.6. The parsimony, unifrac-unweighted and unifrac-weighted tests were applied to determine significance of clustering between the groups in both, OTUs and Phylotypes based dendrograms.
9. Clustering of individuals was also evaluated by plotting the resultant vector of the Principal Coordinate Analysis (PCoA) and by the non-metric multidimensional scaling (NMDS) with 2 dimensions. The R! software was used to generate figures. Analysis of molecular variance (AMOVA) was used to test if the distance between the centers of the clouds of the two groups was greater than individual variation among samples. The correlation of the relative abundance of each OTU with the two axes in the NMDS dataset was calculated in order to determine which OTUs or Phylotypes were responsible for shifting the samples along the two axes. Finally, the Metastats program (13) through MOTHUR was used to identify statistically different OTUs or Phylotypes among groups.
10. Comparison of bacteria between the groups at different phylogenetic levels was performed by using an unpaired t-test after data had been normalized to values between 0 and 1 using MG-RAST.
11. Species-level identifications were sought in order to determine the presence of selected zoonotic and periodontal disease-associated bacteria within the samples. Following initial identification of selected species in MG-RAST (max. e-value 30, min. percent identity 97, min. alignment length 50), the NCBI Basic Local Alignment Search Tool (BLAST) was used to confirm the species-level identity using the nucleotide collection (nr/nt) database (Altschul et al., 1990). Taxonomy was confirmed if the maximum identity of the sequence reported by NCBI BLAST was ≥97% and was the greatest of all listed matches.

Results:

The swine nasal microbiome is complex and highly variable between individuals, with marked variation at the Phylum level. Species richness is high, with between 25 and 277 species per animal (mean 120, median 98). There was no difference in richness (number of different bacterial species) between MRSA carriers and non-carriers (P=0.94).

Overall, the Proteobacteria Phylum was most abundant; however, this varied between individuals.

Firmicutes was the second most common Phylum overall and was predominant in some individuals (largely based on high abundances of *Staphylococcus*, *Bacillus* and *Paenibacillus* spp). The following other phyla were identified in decreasing relative abundances: Bacteroidetes, Spirochaetes, Actinobacteria, Cyanobacteria, Thermotogae, Tenericutes, Synergistes, Fibrobacteres, Fusobacteria, Elusimicrobia, Lentisphaerae and Deferribacteres.

The microbial population structure did not differ between groups (Parsimony test $P>0.05$). Using principal component analysis, there was no apparent clustering of the MRSA carriers and non-carriers. There were few differences between groups at lower taxonomic levels, with no differences at the Phylum (Figure 1) or Class level. There were a few statistically significant differences, including Sphingobacteriales (Bacteroidetes Phylum, $P=0.005$), Burkholderiales order ($P=0.037$) and Comanadaceae family ($P=0.036$)(Proteobacteria Phylum) and *Microbacterium* Genus ($P=0.021$, Actinobacteria Phylum). The relevance of these is unclear, and given the large number of comparisons that were performed and the lack of precedence indicating a potential role of these in influencing staphylococcal colonization, these may be of no biological relevance. However, they should not be dismissed and further study may be indicated.

There was abundant staphylococcal diversity, with a total of 12 different species identified; *S. aureus, S. epidermidis, S. equorum, S. fleuretti, S. hominis, S. pseudintermedius, S. kloosii, S lentus, S. lugdunensis, S. pasteuri, S. saprophyticus* and *S. schleiferi,*

While there was no difference between farm-matched MRSA positive and negative pigs, there was a significant difference between those pigs and pigs from a different farm that were liquid diet-fed (Figure 2). There were significant differences between groups for the three main Phyla (Figure 3). Liquid-fed pigs had significantly fewer Bacteroidetes ($P=0.042$) and Proteobacteria ($P=0.027$), and more Firmicutes ($P=0.004$). There were numerous differences between groups at lower taxonomic levels, including Paenibacillaceae ($P=0.007$), Staphylococcaceae ($P=0.043$), Planococcaceae ($P=0.025$) and Enterococcaceae ($P=0.038$). This indicates that management factors can influence the nasal microbiome, so further comparison of factors that modify the nasal microbiome and the corresponding influence on MRSA (as well as other bacteria that are swine or zoonotic pathogens) is indicated.

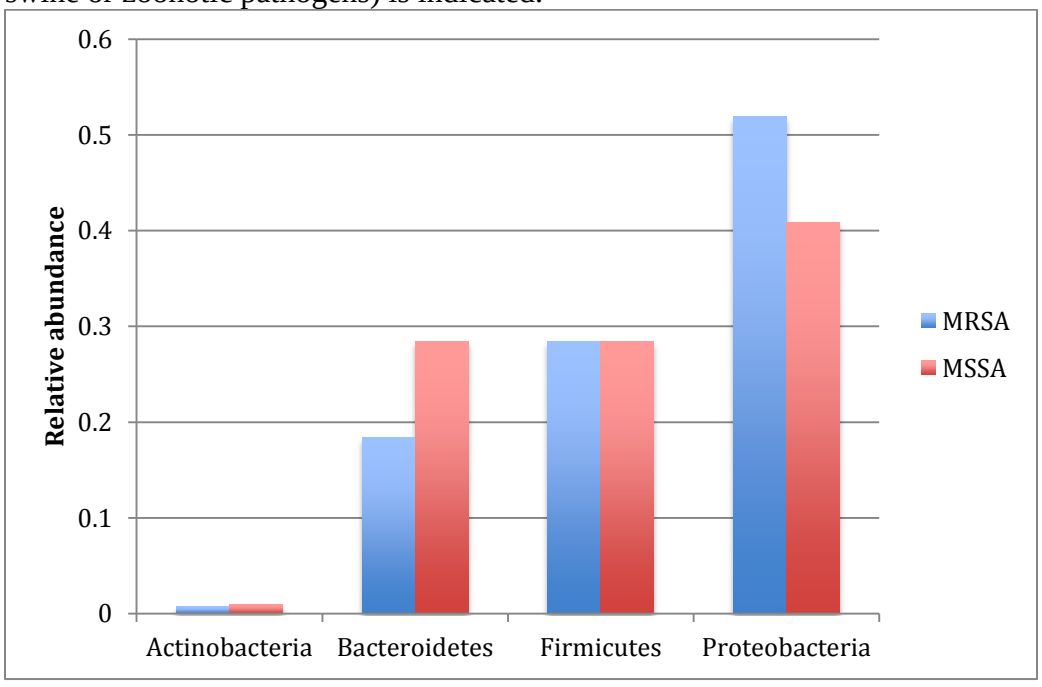

Figure 1: Comparison of the relative abundance of the main bacterial phyla in pigs carrying MRSA compared to farm- and age-matched MRSA negative pigs.

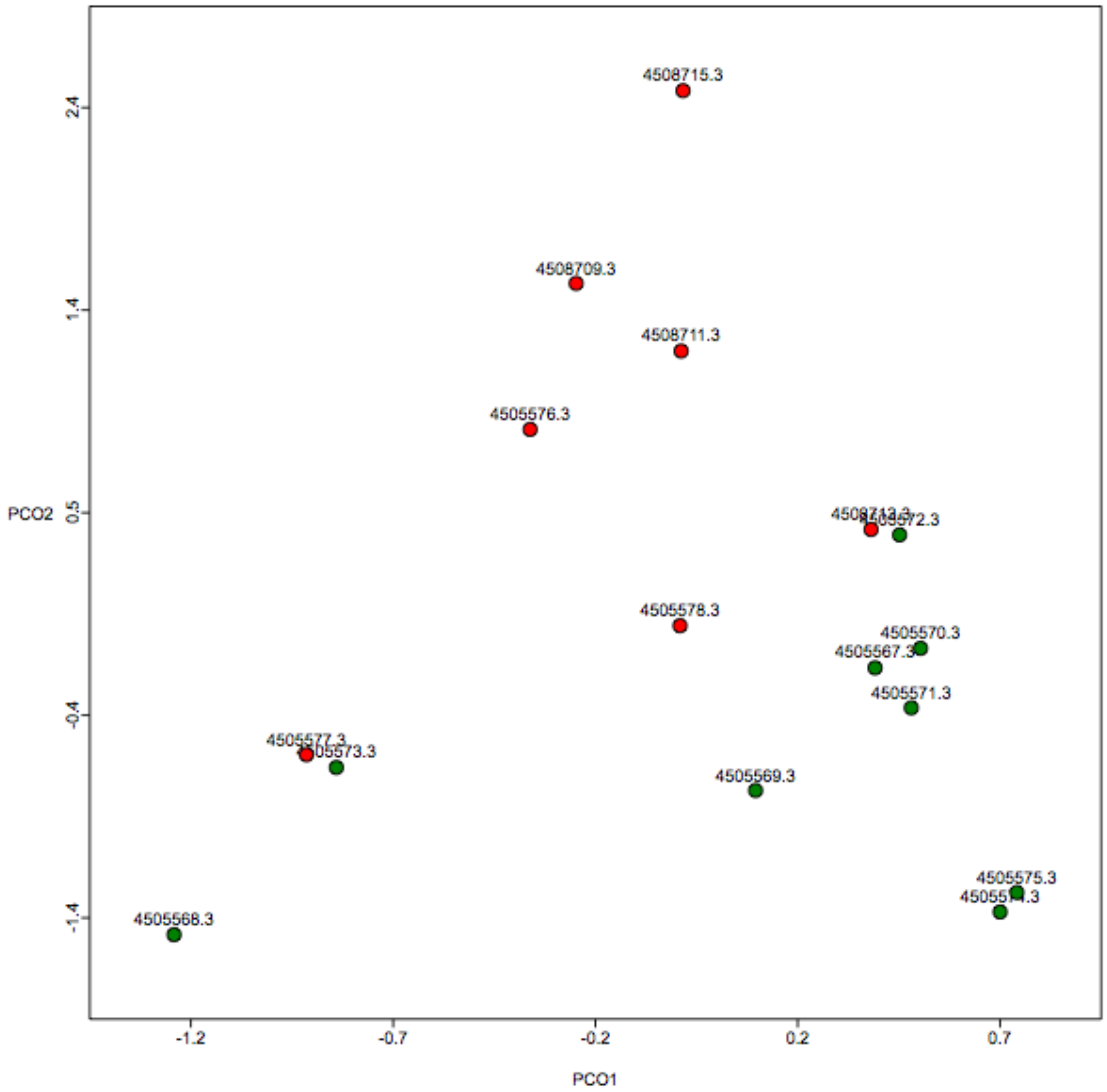

Figure 2: Principal component analysis comparing the nasal microbiome of conventionally-fed pigs (red) versus age-matched liquid diet-fed pigs (green).

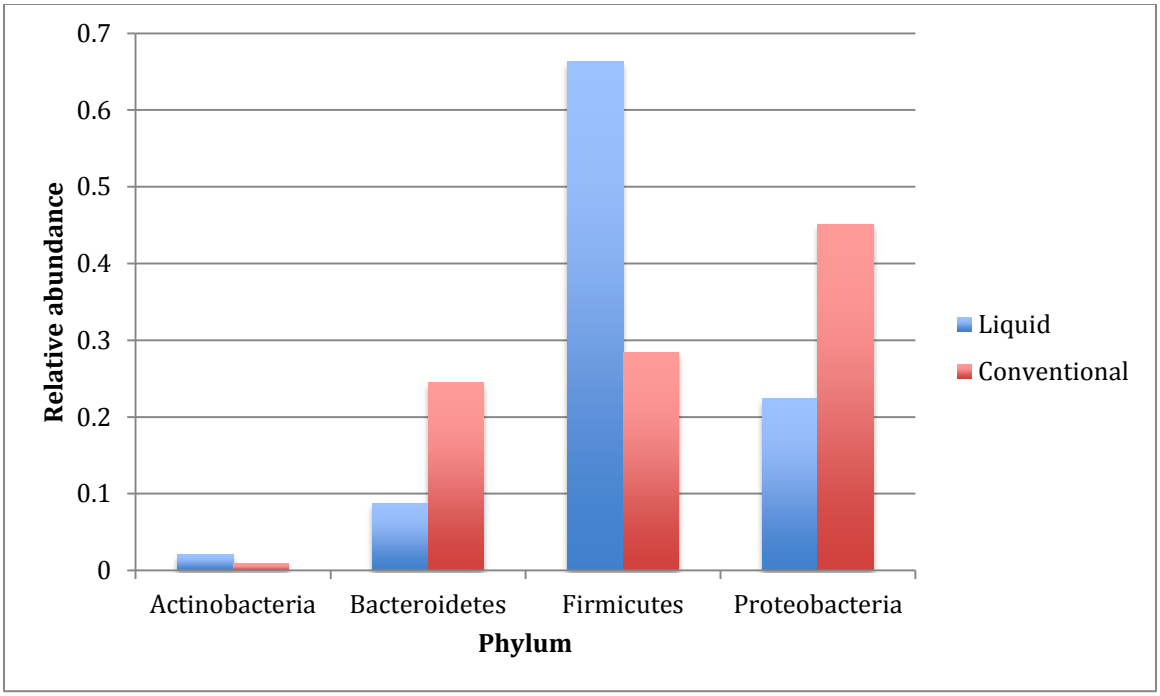

Figure 3: Relative abundances of the main bacterial phyla between different feeding types.

Discussion:

This study has demonstrated that the nasal bacterial microbiota of the pig is a highly complex microbial population with pronounced variation between pigs. The species richness (number of different species present per animal) was high. This complex microbial population probably plays a critical role in the health of the pig, from various standpoints, including influencing the immune system and potentially with various pathogens that can be found in the pig's nasal passages. However, no remarkable differences were present in the nasal microbiota of pigs carrying MRSA versus those not carrying this bacterium. Accordingly, there is no evidence to support the hypothesis that the endogenous nasal microbiota is a key factor in determining whether MRSA will be able to colonize an individual pig, and decreases the likelihood that modification of the nasal microbiota will be an effective tool for MRSA decolonization in pigs. Therefore, other measures to control this concerning bacterium are indicated.

While there was no difference between MRSA carriers and non-carriers, there was a pronounced difference in the nasal microbiome of pigs fed a conventional compared to those fed a liquid diet. The clinical relevance of this difference is unclear but it is possible that differences in the nasal microbiome could influence various aspects, such as the immune response and carriage of various bacterial and viral swine and zoonotic pathogens. This study has provided critical baseline information about the composition of the nasal microbiome and future study of the role of this microbiome (as well as the microbiomes of other body sites) is indicated.