A mammalian commensal of the oropharyngeal cavity produces antibiotic and antiviral valinomycin in vivo

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Abstract

Around weaning, piglets are susceptible to infection by bacterial pathobionts, leading to increased morbidity and mortality. We identified isolates of *Rothia nasisuis* in the upper respiratory tract of weaned healthy piglets that produce valinomycin in vitro and in vivo via its *vlm*-encoded non-ribosomal peptide synthase (NRPS) enzyme complex. Valinomycin is an antiviral and antibiotic ionophore that shuttles potassium ions across membranes and is capable of inflammasome activation and apoptosis in LPS-primed macrophages at concentrations of 1 uM. Polarized monolayers of epithelial cells were much less sensitive to valinomycin but concentrations $\geq$ 10 µM decreased trans-epithelial resistance. *R. nasisuis* inhibited growth of closely related species of *Rothia*. Deliberate inoculation of valinomycin-producing *R. nasisuis* into newborn piglets suggested this species can shape the microbiota post weaning. Our findings support the idea that valinomycin is a competitive niche factor potentially also compromising epithelial integrity to gain access to (micro)nutrients.

Introduction

Host microbiomes and their rich variety of bioactive molecules, impact on host health at all stages of life and are important targets for disease prevention (D'Souza et al., 2018; Sharon et al., 2014). The palatine tonsils function as a secondary lymphoid organ and play an important role in the induction of mucosal antibody responses to commensal and pathogenic microbes. The tonsils are colonized by an extensive microbiota, mainly bacteria which interact with each other and the host, through their metabolites, influencing health and physiology. Porcine tonsils can harbor pathogens such as swine influenza virus and zoonotic pathogens such as *Streptococcus suis* and *Salmonella enterica*, all of which can cause invasive disease. Thus, the composition of the tonsil microbiota can increase susceptibility for infection, by influencing host physiology or by creating a synergistic habitat for pathogens (Cilloniz et al., 2016; Sassone-Corsi and Raffatellu, 2015). Conversely, microbial communities may also benefit the host by providing colonization resistance against pathogens through the production of peptides or specialized metabolites with antimicrobial activity, and by stimulating innate immunity and the secretory antibody repertoire (Buffle and Pamer, 2013; Sassone-Corsi and Raffatellu; Van der Waaij et al., 1971). It is therefore of interest to identify commensal bacteria and their secreted products that inhibit or kill pathogenic microbes.

To discover antibiotic potential of molecules secreted by tonsillar microbes and better understand microbe-microbe and host-microbe interactions within the microbiota associated with this important lymphoid organ we have isolated and characterized over 10,000 single bacterial colonies sampled from the tonsils and small intestine of over 30 healthy piglets. From an initial list of nearly 100 colonies identified in pathogen inhibition screens we selected 2 isolates which reproducibly retained antimicrobial activity after the extracts were protease treated or heated to 100 °C. Phylogenetic analysis of the full-length 16S rRNA gene identified both isolates as *Rothia nasisuis*. Both isolates produced valinomycin, a cyclic dodecadepsipeptide ionophore with potent antibiotic and antiviral properties that is also produced by soil-dwelling *Streptomyces* species, well-known antibiotic producers. We investigated the potential
antibiotic activity of valinomycin on commensals and pathogens that commonly colonize the tonsils, as well as cytotoxicity to host epithelial and immune cells typically found in the tonsillar tissues. *R. nasisuis* is a common species in the oral microbiota of pigs which led us to hypothesize that it might impact on the composition and succession of microbiota before and after weaning. To investigate this, we introduced *R. nasisuis* in the oral cavity of 2-days-old piglets and sampled tonsillar microbiota at 5 timepoints for microbiota profiling during the first 5–6 weeks of life. Our findings suggest that valinomycin is a niche factor that aids *R. nasisuis* colonization in the oropharyngeal cavity through effects on both the host and other members of the microbiota.

**Results**

**Robotics-supported culturing of tonsillar and intestinal samples identified isolates of *Rothia nasisuis* producing heat-stable and protease-resistant bioactives with antimicrobial activity**

Over 10,000 colonies were screened for their capacity to inhibit growth of a panel of bacteria (see Supplementary Methods and Supplementary Table S1) using an overlay assay. Out of nearly 100 colonies with consistent inhibitory activity against pathogens we selected 2 tonsil isolates #32 and #4 for further characterization because their antimicrobial activity was heat-stable and protease resistant. The DNA sequence of the full-length 16S rRNA gene of the isolates had 99.59% identity to *Rothia nasisuis*, a species that has been recently identified in intranasal samples of healthy pigs from Germany (Schlattmann et al., 2018).

**The genomes of *R. nasisuis* isolates #4 and #32 encode an NRPS biosynthetic gene cluster**

The genomes of *R. nasisuis* tonsil isolates (#4 and #32) were almost identical and predicted by antiSMASH 4.0 to encode a single 21.5 kb biosynthetic gene cluster (BGC) belonging to the nonribosomal peptide-synthetase (NRPS) family (Fig. 1A). Substrate specificity of the adenylation (A) domains within orf1 and orf2 was predicted *in silico* from the amino acid sequence (Röttig et al.; Stachelhaus et al.) and by phylogenetic comparisons to the distantly related valinomycin-producing BGCs from *Streptomyces tsukubaensis* and *Streptomyces fulvissimus* and the cereulide biosynthetic gene cluster from *Bacillus cereus* (see Supplemental Information, Figure S1 and S2) (Alonzo et al., 2015; Jaitzig et al., 2014; Magarvey et al., 2006). Assuming that this synthetase follows the general linearity principles of vlm NRPS clusters, it was predicted to form three tetradepsipeptides with the subunits dHiv, dVal, ILac, lVal, as monomers or as dipeptides, which then undergo cyclization by macrolactonization by the terminal thioesterase (TE) domain (Cheng, Y.-Q., 2006; Jaitzig et al., 2014; Magarvey et al., 2006; Marxen et al., 2015). The orfA gene lies upstream of orf1 and orf2 and encodes a discrete type II thioesterase (TEII) that displays 49% identity to the *S. tsusimaensis* TE protein sequence (Cheng, Y.-Q., 2006), and may have a similar function in optimizing NRP assembly by hydrolyzing mis-primed substrates from T domains (Li et al., 2015; Schwarzer et al., 2002). The proteins encoded by orfB and orfC are predicted to function as an ATP-binding protein and an ABC transporter permease, respectively. BLAST searches with downstream OrfB and OrfC protein sequences against a manually curated bacterial ABC transporter database and the
MIBiG database (Medema et al., 2015) identified highest identity with *Bacillus* proteins CesC and CesD, respectively (Fichant et al., 2006). CesC and CesD are annotated as putative ABC transporters associated with the plasmid borne cereulide synthetase cluster from *Bacillus cereus*, and were shown to be essential for export and biosynthesis of cereulide (Ehling-Schulz et al., 2006; Lucking et al., 2015). Interestingly, the encoded protein OrfB shows 44.4% identity and 65.5% similarity to CesC protein sequence (YP_009080546.1) and OrfC shows 29.7% identity and 65.8% similarity to CesD protein sequence (YP_009080547.1); OrfC also bears the same predicted six transmembrane helical regions typical for an ABC transporter permease (Supplemental Information Figure S3) (Fath and Kolter, 1993; Tsirigos et al., 2015). No genetic elements involved with regulation of expression of the NRPS cluster could be detected in the DNA sequence flanking the operon.

The NRPS cluster in *R. nasisuis* has a GC content of 48.7%, which is markedly lower than the overall GC content of the genome (57.98%), calculated by GC-Profile (Gao and Zhang, 2006) (Fig. 1B). The NRPS cluster is located between chromosomal genes *disA* and *radA* in *R. nasimurium*, *R. mucilaginosa*, *R. dentocariosa* and *R. aeria* species lacking the NRPS, *disA* is inverted possibly due to recombination as a result of lateral gene transfer (Fig. 1C).

**The antimicrobial compound produced by *R. nasisuis* isolates #32 and #4 is valinomycin**

Antimicrobial activity was detected in the 70% isopropyl alcohol (IPA) and 0.1% trifluoroacetic acid (TFA) solubilized extracts of isolates #4 and #32 and to a greater extent in the 100% methanol extract (MeOH) (see Supplementary Methods). For purification dried methanol extracts were suspended in diethyl ether (Et₂O), centrifuged, filtered, evaporated to dryness under a vacuum and finally re-extracted in acetonitrile (MeCN). The extracted compounds were purified by reverse-phase HPLC and characterized by MALDI ToF MS and ¹H and ¹³C nuclear magnetic resonance (NMR) (Supplemental Information, Figures S4-S7). Two of the purified compounds had Mass-to-Charge Ratio (m/z) [M + H⁺] of 1111.63 and 1125.47 and identical HPLC retention times and ¹H and ¹³C NMR spectra to commercially sourced valinomycin (Supplemental Information Figure S5 and S6). Based on these results we concluded that our *R. nasisuis* isolates produce the dodecadepsipeptide valinomycin.

LCMS/MS analysis of extracts from *R. nasisuis* isolates #4 and #32 cultured on BHI agar plates confirmed the production of valinomycin (Fig. 2). Furthermore, imaging mass spectrometry showed that valinomycin is secreted into the media around the colonies during colony growth. Molecular network representation of MS/MS spectra from the extracts showed the presence of an additional derivative i.e. montanastatin, a cyclodepsipeptide with the structure, cyclo(⁶Hiv⁵Val⁴Lac⁸Lac⁸Val)₂. No antimicrobial activity has been observed for montanastatin (Pettit et al., 1999) (Fig. 2). Both montanastatin and valinomycin are produced by soil-dwelling *Streptomyces anulatus* (Pettit et al., 1999). Our study appears to be the first report of valinomycin production by a host associated *Rothia* species.

**Valinomycin inhibits Streptococcus and Rothia species occupying the same host niche**
The valinomycin extract from *R. nasisuis* was tested for antimicrobial activity against several Gram-positive species present in tonsil microbiomes as well as Gram-negative *Escherichia coli* and *Salmonella enterica* serovar enteritidis (Table S1) using agar well diffusion assays and colony overlay assays. Growth of *Streptococcus suis*, *Streptococcus mitis*, *Streptococcus parasuis*, and *Streptococcus porcinus* but not *Staphylococcus aureus* was inhibited by valinomycin-producing *R. nasisuis* (Figure S8). All strains and serotypes of *S. suis* were sensitive to valinomycin extracts from *R. nasisuis* (Figure S8). No activity was found against the Gram-negative bacteria tested.

Purified valinomycin was also tested for growth inhibition of *R. nasimurium* DSM15694, the valinomycin-producing *R. nasisuis* isolates #4 and #32 and different strains of *S. suis* using a microbroth dilution assay. Growth of *R. nasimurium* and *S. suis* was inhibited at a valinomycin concentration of 1 µM, whereas growth of isolates #4 and #32 were only partially inhibited at 100 µM (Fig. 3). The MIC of valinomycin for growth of other *Rothia* species found in the tonsil microbiota, namely *R. mucilaginosa* and *R. dentocariosa* were 1 µM and >100 µM respectively (data not shown).

**Valinomycin-positive strains of Rothia and valinomycin are detected in vivo**

As *Rothia* species are frequently identified in porcine oral microbiota (Murase et al.; Pena Cortes et al., 2018) we investigated whether valinomycin-producing *Rothia* strains were present in tonsil swabs of piglets using PCR. We designed one PCR primer set (FLANK) to amplify a genomic DNA region spanning the *vlm* cluster and upstream region, and a second set of primers (INT) to generate an amplicon within the *vlm* cluster (Fig. 1A). The *vlm* amplicons were detected in the DNA extracted from 17 of 21 (80%) tonsil swabs (Table 1). The microbiota composition of these tonsil samples was determined using 454 pyrosequencing to confirm that a high abundance of *R. nasisuis* 16S rRNA gene sequences (OTUs) coincided with the high frequency of presence of the *vlm* gene cluster (Table 1). We found a positive correlation between the quantity of flank *vlm* amplicon (Pearson R = 0.556, p < 0.001) and the internal *vlm* amplicon (Pearson R = 0.469, p < 0.005) with the relative abundance of microbiota reads with >99% identity to the valinomycin-producing *R. nasisuis*, respectively. In addition, PCR analyses confirmed that the valinomycin gene cluster was detectable in the total DNA purified from porcine tonsil swab samples from which the valinomycin-producing strains *R. nasisuis* #4 and #32 were originally isolated.
To determine whether valinomycin was produced in vivo, we extracted metabolites from the original tonsil swabs for LC-MS/MS. Valinomycin was detected in high concentrations in five tonsil swabs (43, 48, 55, 63 and 79 Table 1) which also yielded high amounts of the FLANK and INT NRPS amplicons using PCR. Additionally, four of these samples had a high relative abundance of sequence reads with >99% identity to *R. nasusis* 16S gene sequence. Valinomycin was detected in low amounts in only two other tonsil swab samples (66 and 78), which yielded low amounts of the INT PCR product (Table 1). These data confirmed that valinomycin is present in the porcine tonsil, and that the amount of valinomycin positively correlates with the relative abundance of *R. nasusis* and vlm gene cluster.

Valinomycin induces NLRP3-dependent secretion of IL1β in LPS primed ex vivo bone marrow derived macrophages

Table 1. Detection of *R. nasusis*, the vlm gene cluster and valinomycin in porcine tonsil swabs. The percentage of 454 pyrosequencing reads in sampled porcine tonsils with >99% identity to *R. nasusis* 16S rRNA gene are indicated for each sample, colored from largest (dark green) to smallest value (white). Presence of vlm cluster in the *R. nasusis* genome as determined by PCR, semi-quantified from high (+++, dark red), medium (+, orange) and low (+/-, light yellow) to absent (-, light blue) by intensity of the band corresponding to the expected amplicon on an agarose gel. The LCMS/MS detection of valinomycin is here semi-quantified from high (+++), medium (++), to low (+) as measured by the area of the main peak at m/z = 1128.66.

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Valinomycin induces NLRP3-dependent secretion of IL1β in LPS primed ex vivo bone marrow derived macrophages

Given that valinomycin is reported to be a potassium ionophore and potassium efflux has been suggested to induce the inflammasome pathway (Muñoz-Planillo et al 2014). We tested whether valinomycin would promote IL-1b release and inflammasome assembly in LPS primed WT and Nlrp3<sup>−/−</sup> bone marrow derived macrophages (BMDM). Valinomycin stimulation triggered Nlrp3-dependent IL-1β secretion at 4 and 18 hours (Fig. 4A, B). Accordingly, ASC specks, micrometer-sized perinuclear protein
aggregations indicative of inflammasome assembly, were observed in valinomycin stimulated WT BMDMs, whereas \textit{Nlrp3-/-} BMDMs rarely displayed ASC specking (Fig. 4C, D). This finding indicates that valinomycin is capable of stimulating the production of the proinflammatory cytokine IL-1\(\beta\) in a Nlrp3-dependent manner.

**Differential cytotoxicity of valinomycin for epithelial cells and leukocytes**

In epithelial cells, Na\(^+\)/K\(^+\) ATPase enzyme activity and homeostasis of intracellular K\(^+\) are required for tight-junction formation (Rajasekaran et al., 2001). Thus, we investigated whether the K\(^+\) ionophore valinomycin might affect the barrier function of the epithelium through altered tight-junction formation or cell apoptosis. When 10 \(\mu\)M valinomycin was added to a confluent monolayer of porcine tracheal epithelial cells (ECs), the transepithelial resistance (TER) gradually decreased over 12 to 24 h, indicating a loss of barrier integrity (Fig. 5A). Incubation of polarized ECs with 10 \(\mu\)M valinomycin for 18 hours led to a modest but significant decrease of 15\% of neutral red uptake indicating that the viability or transport capacity of ECs had been reduced upon addition of valinomycin (Supplemental Information Figure S9).

As macrophages, dendritic cells and lymphocytes are commonly found in the tonsillar crypts and associated lymphoid tissue we also tested their sensitivity to apoptosis via valinomycin. To do this we exposed freshly isolated human peripheral blood mononuclear cells (PBMCs) to different concentrations of valinomycin for 18 hours. We observed a significant dose-dependent effect on cell viability and increased numbers of apoptotic cells at valinomycin concentrations of 1 nM and higher (Fig. 5B), indicating that PBMCs are substantially more sensitive to the cytotoxic effects of valinomycin than ECs.

**Longitudinal colonization studies in newborn piglets confirm horizontal transfer of valinomycin-producing \textit{R. nasisuis}**

To investigate the potential for the valinomycin-producing \textit{Rothia nasisuis} strain \#32 to colonize newborn piglets we performed a colonization study in newborn litters of piglets (see supplementary information for further details). Briefly, 3 sows were selected which were farrowing on the same day and the litters of 12 newborn piglets randomized between the three sows A, B and C to avoid genetic bias. The sows were housed in separate maternity pens and approximately 10\(^9\) CFU of \textit{R. nasisuis} strain \#32 was administered by syringe in the oral cavity of piglets nursed by sows A and B at the time points post-parturition shown in Fig. 6, whereas the control litter housed with sow C were not inoculated with \textit{R. nasisuis}. Three of the piglets nursed by sows A and B (i.e. A1-A3, B1-B3) were uninoculated to investigate potential horizontal transmission of \textit{R. nasisuis} strain \#32 using a strain-specific qPCR developed for the valinomycin gene cluster (see supplementary information).

In all new-born piglets from sows A, B and C the copies of the \textit{R. nasisuis vlm} amplicon were below or close to the detection limit for the first 9 days of suckling (< 2 \(\times\) 10\(^2\) copies/mL) (Fig. 7). Therefore, we re-inoculate the piglets housed with sows A and B with \textit{R. nasisuis} on day 19, and again two days after weaning (i.e. d29) due to the reported effects of weaning on microbiota composition. After the second inoculum with \textit{R. nasisuis}, the target copies of the \textit{vlm} amplicon were between 6 \(\times\) 10\(^2\) and 3 \(\times\) 10\(^4\) copies/mL in 11 of the piglets from sow A and between 4 \(\times\) 10\(^2\) and 1 \(\times\) 10\(^3\) copies/mL in 6 of the piglets
from sow B. Between 1 and 3 of the uninoculated piglets fostered by sow A and B also became colonized with *R. nasisuis vlm* on day 20 and, or day 35 suggesting transfer of valinomycin-producing *R. nasisuis* between piglets or from the environment. On day 11 the *vlm* amplicon was also detected in three piglets in the uninoculated group of piglets from sow C, indicating that *vlm*+ *Rothia* was naturally present in the farm and horizontally transferred to some piglets. By day 35, 7 days after weaning and one-week after the last inoculation, valinomycin-producing *R. nasisuis* were detected in 3 to 6 piglets of each litter (Fig. 7). Taken together these results indicate that the valinomycin-producing *R. nasisuis* present on the farm can naturally colonize piglets and that oral inoculation of the strain can increase the number of piglets in which the strain can be detected by qPCR (> 102 gene copies per ml of extracted tonsil swab DNA).

Illumina sequencing of the V3-V4 region of the 16S rRNA gene from bacterial DNA isolated from tonsil swabs was used to assess whether inoculation of valinomycin-producing *R. nasisuis* influenced microbiota composition. In the suckling period the most abundant species were *Streptococcus suis, Fusobacterium gastrosuis, Glaeserella parasuis, Rothia nasimurium*, and *Veillonella spp*. Piglets housed with the same sow had a more similar microbiota composition than piglets housed with different sows (Fig. 8B). For example, on day 20 during the suckling period, the *R. nasisuis* inoculated litters housed with sows A and B showed differences in the relative abundance of *Moraxella plurianimalium* and *Veillonella spp*. In agreement with a previous study (Pena Cortes et al., 2018) changes in microbiota composition were observed during the transition from suckling to weaning. For example, comparing day 20 (suckling period) with day 35 (8 days after weaning) shows decreased relative abundance of *Fusobacterium gastrosuis* and an increase in *Streptococcus porcorum* (Fig. 8A).

Principal coordinate analysis (PcoA) of the microbiota composition of piglets inoculated with *R. nasisuis vlm* (sows A and B) or uninoculated (sow C) shows that all litters had a different microbiota composition on day 20 of the suckling period. Despite the variability in persistence of the valinomycin producing strain in the inoculated piglets, microbiota composition became more overlapping on day 29 (2 days after weaning), and on day 35 (one-week post-weaning) the microbiota composition on the tonsils of piglets from sows A and B that had been inoculated with valinomycin-producing *R. nasisuis* was highly similar and distinct from the group of uninoculated piglets (Fig. 8B) (PERMANOVA, *p* < 0.001). A redundancy analysis (RDA) plot of the microbiota composition of sow A, B and C on day 35 indicating the most abundant taxa contributing to the variability of the samples is shown in Figure S11. These results suggest that valinomycin-producing *R. nasisuis* might influence the ecology of the tonsil microbiota.

Bacterial species that were significantly increased or decreased in relative abundance in the piglets inoculated with *R. nasisuis vlm* on d35 were identified using Kruskal–Wallis rank sum test with FDR correction. Taxa that significantly decreased in relative abundance in in the groups of piglets inoculated with *valinomycin-producing R. nasisuis* include species of *Campylobacter, Actinobacillus, Streptococcus* and *Fusobacterium whereas* the relative abundance of species of *Veillonella, Lactobacillus and Enterococcus* increased compared to the uninoculated group piglets (Table S2).

**Discussion**
In a study aimed at culturing and screening of the antibacterial activity of representative species of the porcine tonsil microbiota, we identified two *Rothia* isolates producing valinomycin, a non-ribosomally synthesized cyclic peptide. Products of functional NRPSs in *Rothia* have not been described before, although the biosynthetic gene clusters tentatively originating from this genus have been found in human metagenomic data (Donia et al., 2014). We discovered that the purified compounds produced by *R. nasisuis* are identical to commercial valinomycin produced by *S. fulvissimus*. To our knowledge, this is the first time a commensal bacterium from mammalian microbiota has been shown to produce valinomycin and until now, the presence of *vlm* NRPS gene clusters and the production of valinomycin had only been attributed to two soil isolates of *Streptomyces fulvissimus* (Brockmann and Geeren, 1957; Brockmann and Schmidt-Kastner, 1955; Cheng, Y.-Q., 2006; Magarvey et al., 2006; Matter et al., 2009) and isolates belonging to the *Bacillus pumilus* group (Wulff et al., 2002).

In addition to valinomycin (*m/z [M + H]+ = 1111*), we also detected small amounts of two structural variants of valinomycin, with one (+ 14 Da) or two (+ 28 Da) additional CH₃ groups in valinomycin derived from *R. nasisuis* and *S. fulvissimus* (Figure S6 & S7). These same variants of valinomycin were also identified by automated search algorithms in the extract of a *S. fulvissimus* strain (Skinnider et al., 2015). It is not uncommon to find very similar structural variants of cyclododecadepsipeptides that differ in one of the three four residue comprising units.

The GC content (48.7%) of the *vlm* cluster in *R. nasisuis* is much lower than the genomic average of this isolate (ca. 60%; Fig. 1B), indicating a high likelihood of horizontal transfer from another species. The *vlm* clusters in *Streptomyces tsusimaensis* (ATCC 15141) and in *S. fulvissimus* (DSM 40593) are flanked by putative transposase-encoding genes, which could facilitate horizontal gene transfer (Cheng, Y.-Q., 2006; Myronovskyi et al., 2013). It is most likely that the *vlm* cluster in *R. nasisuis* originated from another unknown ancestor because we did not find a transposase in the flanking DNA, and the protein sequences are highly dissimilar to those encoded by the *vlm* genes found in *Streptomyces* species. These results suggest that the valinomycin NRPS biosynthetic pathway has been conserved over long evolutionary time scales, which is unusual considering the highly dynamic nature of NRPS evolution that has generally been observed.

Remarkably, the *vlm* NRPS cluster was detected by PCR in many piglet tonsil swab samples from a farm (19 out of 21) and valinomycin detected in tonsil swabs by LC-MS/MS (Table 1). In previous studies *R. nasisuis* was also found in tonsil swabs from USA (Pena Cortes et al., 2018) as well as nasal swabs collected from pigs on different farms in Spain (Correa-Fiz et al., 2016) suggesting they are common members of the tonsil microbiota. These observations and the reported activities of valinomycin prompted us to study effects of valinomycin on epithelial and immune cells *in vitro*, as well as the impact of deliberately inoculating valinomycin-producing *R. nasisuis* in young piglets on microbiota composition during the first 5 weeks of life.

To investigate potential effects of valinomycin on the host epithelium we exposed polarized monolayers of cells from the porcine upper respiratory tract to different concentrations of valinomycin for 18 h. At
relatively high concentrations (10 uM; 10 x higher than the inhibitory concentration for streptococci) cell viability was minimally affected, although transepithelial resistance was significantly reduced over time. This may be related to loss of the K+ gradient across the cytoplasmic and mitochondrial transmembrane potential, reducing transport functions of the epithelial cells and ATP production by the mitochondria (Nelson and Cox, 2012; S. Moore, 1964; Teplova et al., 2006). Valinomycin might therefore make the nasal and tonsil epithelium leaky so R. nasisuis in biofilms can acquire nutrients and micronutrients such as iron. As previously described (Cullen et al., 2015), we found that valinomycin acted as a signal II stimulus for the Nlrp3 inflammasome and triggered pyroptosis in LPS-primed macrophages. Immune cells were much more sensitive than epithelial cells to cytotoxic effect of valinomycin with around 50% of PBMCs being dead or apoptotic after 18 h exposure to only 1 uM valinomycin. This activity of valinomycin may therefore help to prevent killing of R. nasisuis by phagocytes at inflamed mucosal surfaces.

Valinomycin was previously identified as the most potent inhibitor of 10,000 agents tested against severe acute respiratory syndrome – coronavirus (SARS-CoV) in Vero cells, with an EC50 of 0.85 µM and selectivity index for the virus of 80 (Cheng, Y.Q., 2006; De Clercq, 2006; Wu et al., 2004). Porcine respiratory coronavirus infects piglets of all ages, causing subclinical or mild respiratory disease but pigs generally recover if the pneumonia is not complicated by other infections. The high selectivity for the antiviral activity of valinomycin may impact on the risk for respiratory coronavirus infections in swine and potentially humans or other animals with upper respiratory tract colonization by valinomycin-producing R. nasisuis. In future studies it may be of interest to investigate correlations between abundance of valinomycin and its producers, and the virome composition of the upper respiratory tract.

Valinomycin is also likely to be involved in direct competition between R. nasisuis and bacteria inhabiting the oropharyngeal cavity, particularly in biofilms. We found that valinomycin inhibited growth of the closely related species R. nasimurium, R. mucilaginosa and several species of Streptococcus.

The deliberate oral inoculation of valinomycin-producing Rothia strain into the litters of two separately housed sows 2 days after birth did not lead to efficient colonization of piglets in the first two weeks of suckling when swabs were collected on day 9 (Fig. 7), perhaps because it is not an early colonizer and as shown in humans, the microbiota community changes rapidly in the first few days of life (Hill et al., 2017).

Re-inoculation of piglets fostered by sow A and B on d19 and d27 led to colonization of 6 to 11 piglets by valinomycin-producing Rothia on d29 and 3 to 6 piglets 1 week after the last inoculum (Fig. 7). These findings reveal high variability in the persistence of the inoculated strain among piglets of the litter, which might be due to the fact that differing amounts of potentially competing taxa including R. nasimurium strains were present in the first few days of life (Fig. 7). We also observed that depending on the time point, between 1 and 3 of uninoculated control piglets in litter C were colonized with vlm + R. nasisuis strains. This is consistent with natural colonization and is in agreement with the finding that valinomycin was detected in 5 of 20 piglet swabs using LC-MS/MS (Table 1) from a different farm in the same geographic area.
Despite the variability in persistence of the valinomycin producing strain in the inoculated piglets, PCoA revealed that the tonsil microbiota of the litters from sows inoculated with valinomycin-producing *R. nasisuis* were highly similar and different from the uninoculated group of piglets one week after the last inoculum (Fig. 8B). These findings suggest that the inoculation of the valinomycin-producing *R. nasisuis* can influence tonsil microbiota ecology and warrants further study using larger groups of sows.

The taxa significantly decreased in abundance in the tonsil microbiota of piglets inoculated with valinomycin-producing *R. nasisuis*, included pathogenic species (Table S2) but there was no significant impact on the abundance of *S. suis* despite the strong inhibitory activity against this pathogen *in vitro* (Figure S11). This could be due to the fact that *S. suis* colonises newborn piglets rapidly, reaching $10^7$ genome copies/mL in the DNA isolated from the tonsil swab already during the first days of life, whereas the average abundance of the *Rothia* strain in qPCR-positive piglets was around $10^3$ genome copies/mL (Figure S11). After weaning the amount of *S. suis* decreased about 10-fold in all litters presumably due to competition from the microbiota which increased in diversity after weaning (Figure S11). Recent studies using spectral imaging fluorescence *in situ* hybridization to visualize the human oral microbiome revealed complex structural organization of the constituent organisms at the micron-scale (Mark Welch et al., 2016). Some species evidently interact more closely than others in natural biofilms. Thus, the structure and local composition of the tonsil biofilm community might also determine which species can be inhibited by valinomycin.

Given the lipophilic nature of valinomycin its effects are likely to be short-range, providing a competitive advantage in bacterial biofilms. Valinomycin is likely to be a niche factor (Hill, 2012) that enables *R. nasisuis* to compete with closely related *Rothia* species and other species occupying the same biofilm niche and utilizing the same nutrient requirements. Additionally, on the host side, valinomycin may alter permeability of the colonized epithelium to increase nutrient availability to colonizing bacteria and through its antiviral effects prevent inflammatory responses thereby contributing to ecological stability.

Further efforts are clearly needed to fully understand the role of specialized metabolites like valinomycin in shaping microbial communities. This may also contribute to a better understanding of the establishment of polymicrobial infections and the pathogenesis of diseases (Donia et al., 2014; Donia and Fischbach, 2015). Moreover, the discovery and identification of novel antibiotic-producing members of the microbiota may lead to new strategies to prevent or treat infections and provide molecular templates for the development of new drugs.

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**Figures**

Figure 4

A, B) IL1β secretion by WT and Nlrp3-/- BMDMs treated with different concentrations of valinomycin. A) 4 h incubation and B) 18 h incubation. C) ASC-speck formation (white arrows) after BMDM stimulation with 10 µM valinomycin for 18 h. D) Quantification of speck formation. (For A, C and D n = 3, for B n = 2, mean ± SEM, * indicates P < 0.05 as determined by students T-test, † indicates P < 0.05 as determined by one-way ANOVA and Tukey's Multiple Comparison Test).
Figure 6

Schematic representation of the experimental animal study displaying timepoints for oral inoculum of valinomycin-producing R. nasisuis (black arrows) to 9 of the 12 piglets housed by sows A and B. Red bars indicate days on which tonsil swabs were taken for microbiota and qPCR. On day 2 the tonsil swab was collected prior to inoculation.

Figure 7

Quantification of R. nasisuis vlm+ in piglet tonsil swabs during suckling and post-weaning period. Only values above the detection limit (horizontal dotted line) are plotted in the graphs. Sows A and B (red) fostered piglets inoculated with vlm+ Rothia nasisuis, whereas sow C (green) fostered uninoculated piglets. Data points shown in light red indicate piglets with sow A or B that were not inoculated with Rothia but nevertheless became colonized. Samples from piglet A1 were not collected on d20, d29, and d35 and from piglet A8 on d29. Days (d) on which tonsil swabs were taken for qPCR are indicated. On day 2 the tonsil swab was collected prior to inoculation.