



In vitro Mixed Biofilm of *Streptococcus suis* and *Actinobacillus pleuropneumoniae* Impacts Antibiotic Susceptibility and Modulates Virulence Factor Gene Expression

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Streptococcus suis (*S. suis*) and *Actinobacillus pleuropneumoniae* (*A. pleuropneumoniae*) are primary swine pathogens that have been frequently co-isolated from pigs suffering from severe respiratory disease. The purpose of this study was to investigate the biological impacts of the interactions between *S. suis* and *A. pleuropneumoniae*. A single- and dual-species culture model was established *in vitro* via *S. suis* HA9801 (serotype 2) and *A. pleuropneumoniae* CVCC265 (serotype 1). The single or mixed biofilms were imaged by confocal laser scanning microscopy. The biomass and viable cells in biofilms were quantified by crystal violet staining and determination of colony-forming units. The antibiotic susceptibility was determined by a microdilution broth method. The differences in gene transcription in pure- or mixed-species biofilms of *S. suis* and *A. pleuropneumoniae* was evaluated by quantitative PCR. *S. suis* and *A. pleuropneumoniae* formed two-species biofilms when co-cultured *in vitro*. When co-cultured with *S. suis*, biofilm formation by *A. pleuropneumoniae* was significantly increased with the absence of NAD that is necessary for the growth of *A. pleuropneumoniae*. Moreover, compared with monocultures, the antibiotic resistance of *S. suis* and *A. pleuropneumoniae* was both enhanced in the co-culture model. When grown in dual-species biofilms, for *A. pleuropneumoniae*, genes associated with virulence factors, including exotoxins and adhesins, were significantly upregulated. For *S. suis*, virulence factor-related genes *cps2*, *gdh*, *mrp*, and *sly* were highly induced. These results suggest that the interspecies interactions between *S. suis* and *A. pleuropneumoniae* may be cooperative under specific conditions and may play an important role in the disease progression and persistent infection.

Keywords: *Streptococcus suis*, *Actinobacillus pleuropneumoniae*, mixed biofilm, antibiotic susceptibility, swine

INTRODUCTION

Respiratory diseases constitute the most important health issues affecting the swine industry worldwide and are often referred to as the porcine respiratory disease complex (PRDC) (Qiao et al., 2011). In general, PRDC causes lung lesions, which, in turn, results in substantial economic losses and impaired animal welfare (Fablet et al., 2012). PRDC is multifactorial in nature and is triggered by mixed infections involving primary and secondary pathogens (Cheong et al., 2017). Clinically, *Streptococcus suis* (*S. suis*) and *Actinobacillus pleuropneumoniae* (*A. pleuropneumoniae*) are two important respiratory pathogens frequently detected in the PRDC (Opriessnig et al., 2011). In recent years, there have been many cases of mixed infections associated with the presence of *S. suis* and *A. pleuropneumoniae* in China. It is more than likely that the different bacteria or viruses coexisting in PRDC interact and exacerbate the pathogenesis of the disease. However, few research groups investigated the interactions between *S. suis* and *A. pleuropneumoniae*, except for a study reporting that these two bacterial species form polymicrobial biofilms *in vitro* (Ramirez-Castillo et al., 2018).

The formation of biofilm is considered as a survival strategy for bacterial pathogens. Bacteria in biofilms are embedded in an extracellular matrix, which reduces their susceptibility to antibiotic and host immune system (Vuotto and Donelli, 2019). This is a major reason why biofilm-related infections are difficult to eradicate. During a chronic infection, bacteria are able to switch between planktonic and biofilm lifestyle (Kathju et al., 2009; Lister and Horswill, 2014). The key role of biofilms have been confirmed in many diseases such as endocarditis, periodontitis, and otitis media (Kobayashi et al., 2005). Multi-species biofilms may be the most significant lifestyle of microbes *in vivo* and bacteria in such polymicrobial biofilms are more difficult to eradicate (Wang et al., 2018). In multi-species biofilms, microorganisms will enhance and ensure their survival and reproduction through communication, competition, or cooperation (Yang et al., 2011). In the present study, we hypothesized that there are synergistic interactions between *S. suis* and *A. pleuropneumoniae* in dual-species biofilms, resulting in enhanced biofilm formation ability, enhanced antibiotic resistance, and upregulated virulence factor gene expression.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

S. suis HA9801 (SS) was isolated from a diseased pig in Jiangsu Province, China, and identified to be *S. suis* serotype 2. All experiments in this study were approved by the Experimental Animal Monitoring Committee of Henan University of Science and Technology and carried out accordingly. *A. pleuropneumoniae* CVCC 265 (APP) was purchased from China Veterinary Culture Collection Center (CVCC).

S. suis was grown in tryptic soy broth (TSB) or plated on TSA. *A. pleuropneumoniae* was cultivated in the same media supplemented with 0.1 µg/mL of β-nicotinamide-adenine-dinucleotide (NAD). Bacterial cultures were incubated at 37°C. All experiments described below were replicated in biological triplicate.

Planktonic Growth Assays of Mono- and co-Cultures

Bacterial colonies of *S. suis* and *A. pleuropneumoniae* were inoculated separately in solid media and grown overnight to mid-exponential phase in TSB + 0.1% NAD medium. The bacterial cultures were diluted in fresh medium to obtain an optical density at 660 (OD₆₆₀) of 0.03. Mono-culture of *S. suis* or *A. pleuropneumoniae* and co-culture of mixture (1:1 ratio) were inoculated in TSB + 0.1% NAD medium for 24 h. Samples were taken at 0, 2, 4, 6, 8, 10, 12, and 24 h, serially diluted in sterile PBS, and then plated on TSA or TSA + 0.1% NAD medium to discriminate *S. suis* or *A. pleuropneumoniae*, respectively, and the viable cell counts were enumerated.

For the mixed culture, the Competitive Index (CI) was calculated according to the formula: $(A. pleuropneumoniae/S. suis)_{output}/(A. pleuropneumoniae/S. suis)_{input}$. The output and input samples were assessed by plating onto selective medium at different time points, respectively. The Relative Increase Ratio (RIR) was similar to CI, and was calculated from the corresponding growth results obtained from single cultures of each strain (Macho et al., 2007). A positive CI value suggests a competitive advantage for *A. pleuropneumoniae* and vice versa. Only CIs that are statistically different from the RIRs at the same growth stages can be recognized as the result of prominent competition between species (Macho et al., 2007).

Biofilm Formation by Single and Mixed Cultures

Biofilm production was quantified by crystal violet staining as described previously (Bragonzi et al., 2012). Briefly, overnight cultures of *S. suis* and *A. pleuropneumoniae* were diluted 1/100 in fresh TSB broth supplemented with NAD, and inoculated individually or at several different ratios in 96-well plates. After 24 h incubation, the medium and planktonic bacteria were removed, and each well was gently washed twice with sterile PBS. Methanol was used to fix the attached bacteria for 15 min. The plates were then air-dried and the biofilms were stained with crystal violet (0.1%). After 20 min, the excess dye was discarded and plates were washed twice with sterile PBS prior to adding 200 µl of 95% ethanol to the wells to dissolve the biofilms. The optical density at 620 nm (OD₆₂₀) was measured using a microplate reader.

Biofilms were prepared in the 96-well plates as described above. Biofilms were washed twice with sterile PBS, and then bacteria were detached and homogenized in 100 µl of sterile PBS by weak sonication for 4 min. TSA or TSA + 0.1% NAD medium was used to discriminate *S. suis* or *A. pleuropneumoniae*, respectively, and then viable cells were enumerated (Chan et al., 2017).

TABLE 1 | The primers used in this study for qRT-PCR.

Primer	Forward primer	Reverse primer
APP		
16S rRNA	GGAGCTTGCTTTCTTGGCCGACG	TAACCTTGCGGCCGTACTCCC
Apx-I	TTGAAGCGGAGAAACAGCTT	TGACCGACCTCGATAAAACC
Apx-II	GGTCAAGGAAAT GGAGTTCAAGAT	GCTAGTTTTT GCAATGTCCAA
afuB	TGGTTTTTAACGAACTGCCTTT	CTTTAATGATGCGCCAATGT
hgbA	CGGATCCGTTTAGCTTCTTG	TAATGCGGCTTCTTTCGTCT
Pga	GATAAAGCAAGCCAG TTCTTAGGT	GCTGTTTGATGAG AAATACCGA
Apa-I	TTGCAGCAGGTGACGTGAA	TCGCTGACCGCGTATAATT
SS		
16S rRNA	GTTGCGAACGGGTGAGTAA	TCTCAGGTCGGCTATGTATCG
cps2	ATTGGTAGGCACTGTCGTTGGTC	AGAACTTAGCATTGTTGCGGTGG
fbps	AACCATCTTGCCAGGCTCCAC	CAGTTCAGAAGCCGATCCCCGAC
gdh	CACCTTTACCACCGCCGATTG	GGAAATGTTCAAGTCAACCGTGG
mvp	CAGGTAACATCAGAATCACCA CTTTT	AAGTTTTGTTGAGCATCCT CTATAGC
sly	TCATTGAGGTGCTTATGTTGCG	GAAGA TTGCG AGCAT TTCCT GG

Confocal Laser Scanning Microscopy

Chamber slides were inoculated with bacterial suspension (*S. suis* alone, *A. pleuropneumoniae* alone and combination in 1:1 ratio) for 24 h at 37°C. The slides were washed twice with PBS to remove the medium and unattached bacteria. Samples were stained with SYTO 9 solution following the manufacturer's instructions from LIVE/DEAD™ BacLight™ Bacterial Viability Kit (Thermo Fischer Scientific, Inc., Waltham, MA, United States), and then washed with PBS (Tawakoli et al., 2013). Biofilms were observed using a Zeiss LSM800 CLSM (Carl Zeiss, Jena, Germany).

Antibiotic Susceptibility Testing

The *in vitro* antibiotic susceptibility of *S. suis* and *A. pleuropneumoniae* was determined by a twofold dilution method in microplates following the guidelines of Clinical and Laboratory Standards Institute. Antibiotic serial dilutions were made up in culture medium, and 100 µl was transferred to the wells. Overnight cultures of *S. suis* and *A. pleuropneumoniae* were diluted at 1:100 with TSB contain in 0.1% NAD, and 100 µl of *S. suis*, *A. pleuropneumoniae*, or combination in 1:1 were added to 96-well plates and incubated at 37°C for 24 h. The minimum inhibitory concentration (MIC) values were determined by reading the optical density and visual observation of the turbidity.

The minimum biofilm eradication concentration (MBEC) values were also determined. Biofilms were prepared in the 96-well plates as described above. Wells were washed twice with PBS and antibiotic serial dilutions made up in TSB containing 0.1% NAD were transferred (200 µl) to the wells. The plates were incubated for another 24 h.

TABLE 2 | *In vitro* susceptibility of pure culture and co-culture of *S. suis* and *A. pleuropneumoniae* in planktonic growth and biofilm to seven clinically relevant antibiotics.

Antibiotic	<i>Streptococcus suis</i>	<i>Actinobacillus pleuropneumoniae</i>	<i>S. suis</i> + <i>A. pleuropneumoniae</i>
Tylosin tartrate			
MIC	<0.3125	20	20
MBEC	1.25	40	80
Gentamicin			
MIC	10	10	10
MBEC	10	10	10
Amoxicillin			
MIC	<0.3125	160	>160
MBEC	0.625	160	>160
Apramycin			
MIC	40	80	80
MBEC	40	80	>160
Tilmicosin			
MIC	5	5	10
MBEC	80	80	160
Spectinomycin			
MIC	40	80	80
MBEC	80	80	160
Fosfomycin			
MIC	80	20	20
MBEC	>160	40	>160

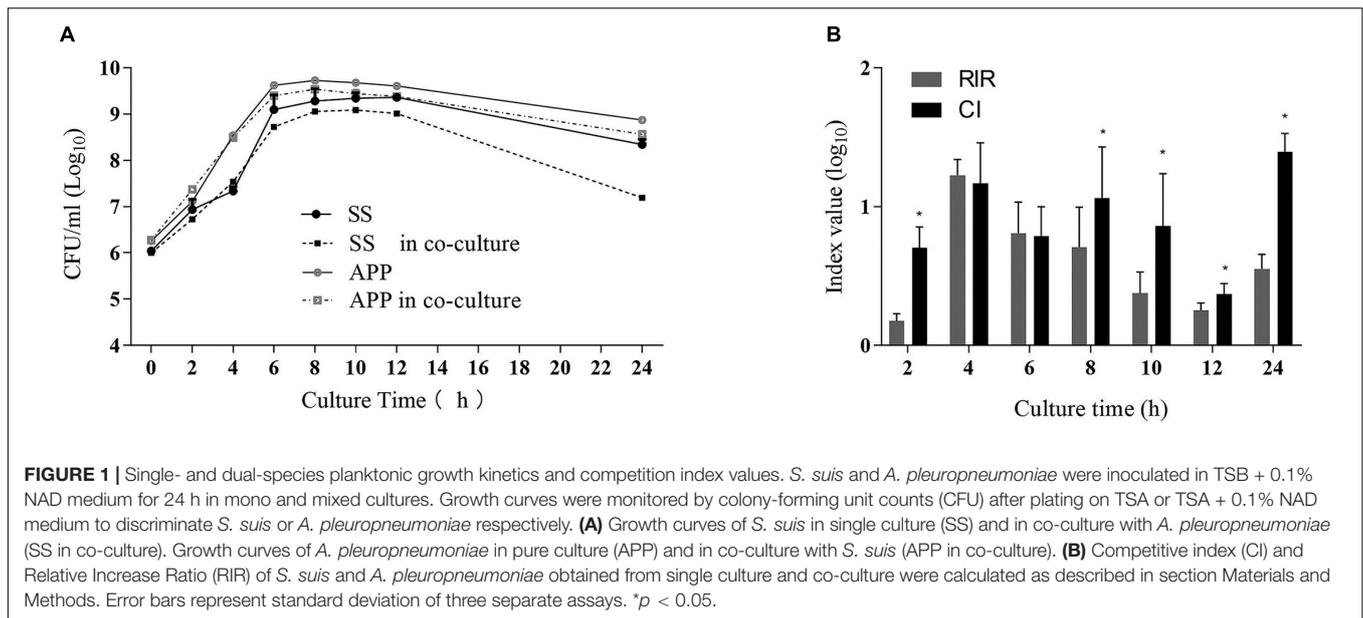
The MBEC values were determined by plating samples on culture medium plates.

Virulence Factor Gene Expression in Mixed Biofilm

Real-time PCR was used to assess the relative expression of virulence factor genes in *A. pleuropneumoniae* or *S. suis* when grown alone or in mixed biofilms. Biofilms were prepared in 24-well plates as described above, washed twice with PBS, detached by sonicating, and then collected. RNA was extracted by the Trizol method (Pompilio et al., 2015). gDNA removal and cDNA synthesis were performed using PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa) according to the manufacturer's protocol. Real-time PCR assay was performed with TB Green® Premix Ex Taq™ (TaKaRa) following the manufacturer's instructions. The primers used are listed in **Table 1**. The 16S rRNA was used as the house-keeping gene for normalization. Relative expression levels were determined by the ($\Delta\Delta Ct$) method.

Statistical Analysis

GraphPad Prism version 7.0 (GraphPad Software, San Diego, CA, United States) was used for data analysis. Results were obtained from three independent experiments and all values were expressed as means \pm standard deviation. Differences between mean values were evaluated by Student's *t*-test. *P*-value of 0.05 or less was considered statistically significant.



RESULTS

Competition Between *S. suis* and *A. pleuropneumoniae* in Planktonic co-Cultures

To study the interactions between *S. suis* and *A. pleuropneumoniae* in planktonic co-cultures, the growth curves of single and mixed cultures were compared, and the results are shown in **Figure 1A**. The kinetics analysis showed that the growth of *S. suis* in mixed culture was negatively affected from 8 to 24 h, while *A. pleuropneumoniae* growth was not clearly affected when co-cultured with *S. suis*. To further estimate the differences in growth kinetics between *S. suis* and *A. pleuropneumoniae* in single or mixed cultures, CI and RIR indexes were calculated. As shown in **Figure 1B**, a positive CI index of *A. pleuropneumoniae* versus *S. suis* was always observed, indicating a competitive advantage for *A. pleuropneumoniae* over *S. suis* in co-cultures. The CI was significantly higher than the RIR between 8 and 24 h ($p < 0.05$), suggesting a noticeable negative effect of *A. pleuropneumoniae* on *S. suis* growth.

Formation of Mixed Biofilm of *S. suis* and *A. pleuropneumoniae*

Biofilm formation by *S. suis* and *A. pleuropneumoniae* in single and dual culture in 96-well plate was assessed by crystal violet staining and viable count, and the results are shown in **Figure 2**. Both *S. suis* and *A. pleuropneumoniae* formed important biofilms when grown in TSB supplemented with NAD (**Figure 2A**). However, in the absence of NAD, *A. pleuropneumoniae* couldn't form biofilms (**Figure 2B**). Under favorable growth conditions (supplement with NAD) for *A. pleuropneumoniae*, mixed biofilms with *S. suis* were formed (**Figure 2C**). Moreover, based on results of crystal violet staining and determination of CFU, *A. pleuropneumoniae* was able to

grow and form a dual-species biofilm without the addition of NAD when grown in the presence of *S. suis* (**Figure 2D**).

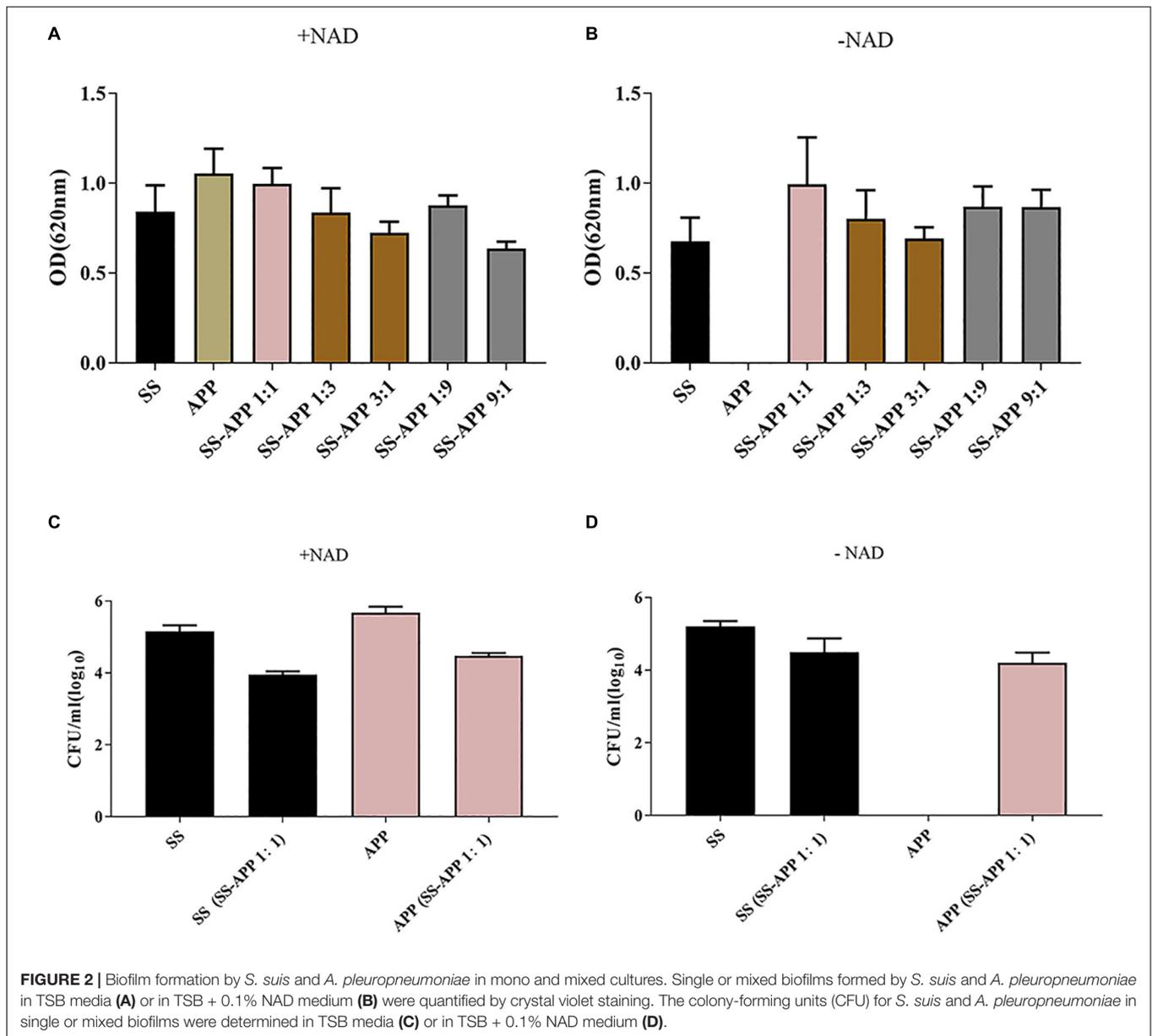
By using confocal laser scanning microscopy (**Figure 3**), it could be confirmed that both *S. suis* and *A. pleuropneumoniae* were able to form robust single- and dual-species biofilms *in vitro*.

Antibiotic Susceptibility

The *in vitro* antibiotic susceptibility of *S. suis* and *A. pleuropneumoniae*, individually and in combination to several antimicrobial drugs, was determined (**Table 2**). For tylosin tartrate, when in co-culture with the other, the MBEC of *S. suis* increased from 1.25 to 80 $\mu\text{g/ml}$, and the MBEC of *A. pleuropneumoniae* increased from 40 to 80 $\mu\text{g/ml}$. For tilmicosin, when in co-culture, the MIC and MBEC of *S. suis* or *A. pleuropneumoniae* were twice as high as that in pure culture. In general, *S. suis* and *A. pleuropneumoniae* in mixed biofilms showed increased resistance to tylosin tartrate and tilmicosin with higher values of MBEC in comparison to single-species biofilms.

Differential Gene Expression in Mono- and Dual-Species Biofilms

As shown in **Figure 4**, in comparison with the single species biofilms, the virulence factor genes in the two-species biofilms were overall upregulated. For *A. pleuropneumoniae*, compared with mono-species biofilms, *ApxI* and *ApxII*, coding for exotoxin, were upregulated by 10.65- and 22.58-fold, respectively, *afuB* involved in biofilm formation was upregulated by 8.69-fold, *apaI* associated with adhesin was upregulated by 5.73-fold, and *hgbA* involved in iron uptake was upregulated by 18.3-fold (**Figure 4A**). For *S. suis*, virulence factor related genes *cps2*, *gdh*, *mrp*, and *sly* were significantly upregulated by 2.61-, 2.23-, 3.81-, and 2.26-fold, respectively, with no statistical differences of

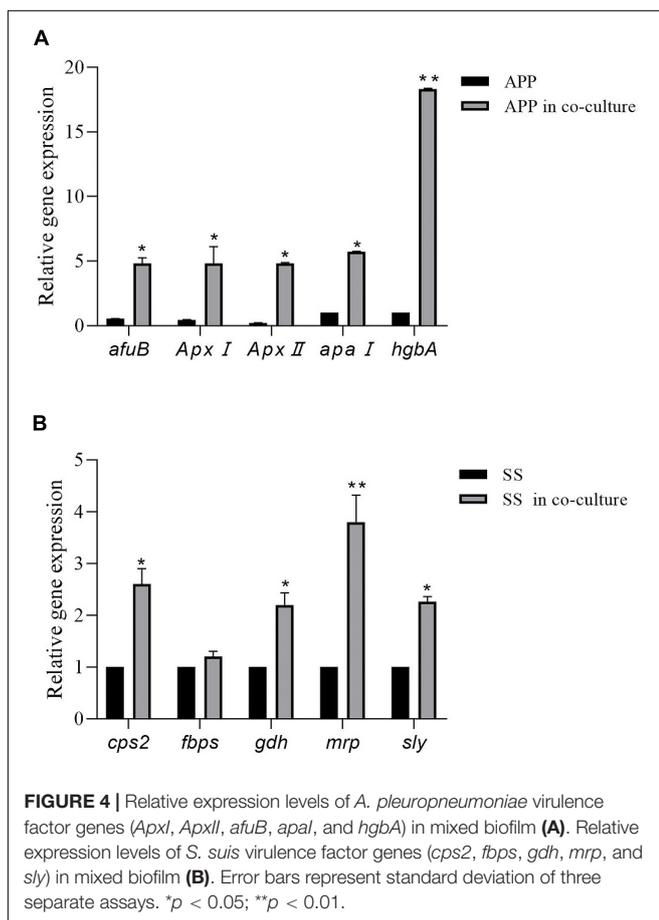
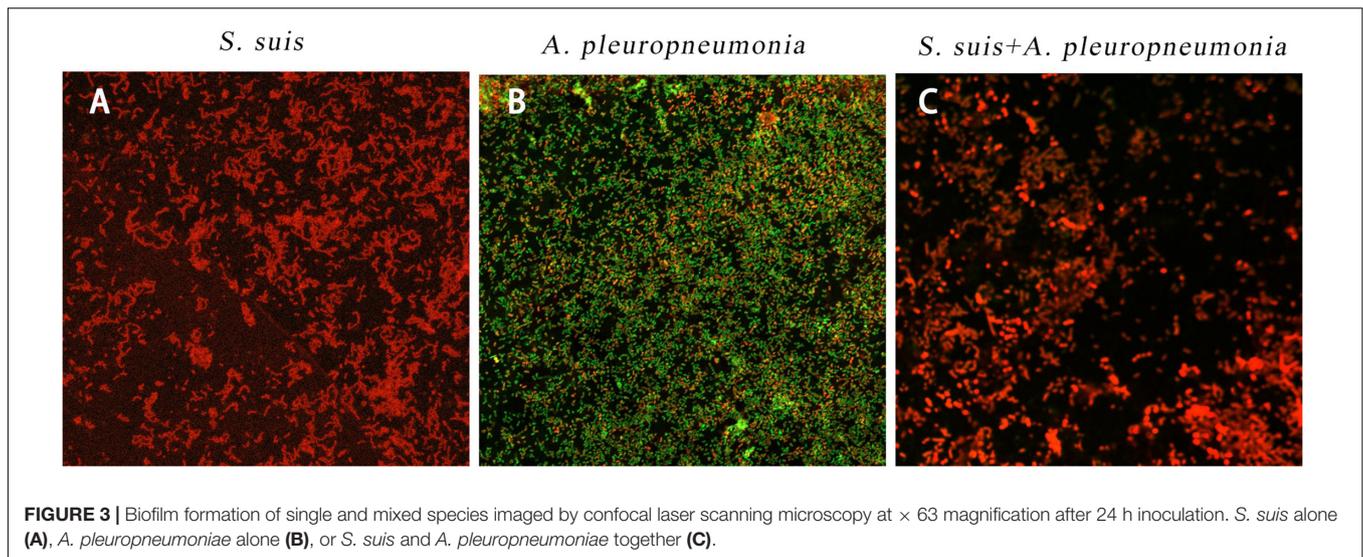


fbps gene (Figure 4B). The results may indicate that co-culture may modulate the bacterial virulence in mixed biofilm.

DISCUSSION

Polymicrobial respiratory diseases remain a major threat in the swine industry worldwide. Pathogens associated with PRDC include swine influenza virus (SIV), porcine circovirus type 2 (PCV2), porcine reproductive and respiratory syndrome virus (PRRSV), *S. suis*, *A. pleuropneumoniae*, *Mycoplasma hyopneumoniae*, and *Haemophilus parasuis*. Bacterial-viral co-infections were reported to exacerbate the pathogenicity (Opriessnig et al., 2011). For instance, co-infections of *M. hyopneumoniae* and SIV lead to the

exacerbation of the clinical signs (Thacker et al., 1999), and during the co-infection of PRRSV and *S. suis*, the virulence of PRRSV was enhanced by *S. suis* and PRRSV increased the susceptibility of pigs to *S. suis* infection (Thanawongnuwech et al., 2000). *S. suis* and *A. pleuropneumoniae* are two important pathogens associated with PRDC, and their co-occurrence in the same site of infection has been frequently reported (Opriessnig et al., 2011), although the interactions between them and the host during coinfection have not been previously investigated. The present study examined the interactions between *S. suis* and *A. pleuropneumoniae* in planktonic and biofilm cultures, and specifically explored whether these interactions provide greater fitness than single cultures, which is helpful to better understand the possible role of coinfection in the pathogenesis.



Studies on microbial interactions under planktonic growth conditions have shown that species can coexist with other microbes competing in the same repository through multiple mechanisms (Hibbing et al., 2010). It was reported that the consumption of limited nutrients may impact the interactions

process between species in co-culture models (Sibley et al., 2008). We demonstrated that *S. suis* has no effect on the growth of *A. pleuropneumoniae* in planktonic culture, while a significant negative impact of *A. pleuropneumoniae* on *S. suis* growth was found during the stationary phase of bacterial growth.

When grown in mixed cultures, the exoproducts of *A. pleuropneumoniae* may be the disadvantageous cause of *S. suis* in planktonic bacteria. The CI and RIR indicated that *A. pleuropneumoniae* surpasses *S. suis* in exponential and stationary phases of bacterial growth. The competition for limited nutrients and space may lead to the antagonistic effects among microorganisms within a community (Harrison, 2007).

Crystal violet staining and colony-forming unit results showed that both *S. suis* and *A. pleuropneumoniae* formed strong biofilms when grown in single or mixed culture, and the CSLM images provided additional evidence. Interestingly, *A. pleuropneumoniae* was able to grow in the absence of NAD when co-cultured with *S. suis*. Our results confirm that the presence of *S. suis* promoted *A. pleuropneumoniae* biofilm growth under a hostile condition for *A. pleuropneumoniae* (without NAD supplementation). A variety of animal or human pathogens, such as *Streptococcus mutans*, *Pseudomonas aeruginosa*, *Legionella pneumophila*, *Stenotrophomonas maltophilia*, and *Escherichia coli*, have been reported that to form mixed biofilms, which help to improve their resistance, persistence, and pathogenicity (Yang et al., 2011). Recent studies on multi-species community behavior have shown that the presence of other bacterial species can modulate the virulence and the gene expression of pathogens. In the respiratory tract, the NAD supply, which is essential for *A. pleuropneumoniae* growth, is rather limited. However, *A. pleuropneumoniae* has overcome this deficiency in various ways, such as cell lysis, which releases nutrients into the surrounding environment (Chiers et al., 2010). In addition, polymicrobial biofilm formation with *S. suis* enables *A. pleuropneumoniae* to acquire this compound by cross-feeding.

We showed that a mixed biofilm of *S. suis* and *A. pleuropneumoniae* has an increased resistance to several

antibiotics. In fact, it is well known that bacteria residing in biofilms have an increased resistance to antibiotics (Assavacheep and Rycroft, 2013), and multi-species biofilm is believed to provide an enhanced protection against antibiotic and host immune system (Armbruster et al., 2010). Some well-known mechanisms for enhancing multi-microbial biofilm resistance include upregulation and transfer of drug resistance genes, increased responsiveness through quorum sensing, and increased mutation level in antibiotic target molecules (Hoiby et al., 2010). Recent research of multi-species community behavior have reported that the virulence, biofilm formation, and the gene expression level of pathogens can be regulated by the presence of other species (Duan et al., 2003). To investigate this, quantitative PCR was performed to analyze differential gene expression in mixed biofilms. The RT-PCR results showed that genes of *A. pleuropneumoniae*, coding for exotoxin, biofilm formation, or iron uptake, respectively, were highly induced. Further, genes of *cps2*, *gdh*, *mrp*, and *sly* associated with virulence factors of *S. suis* were significantly upregulated. The result suggests that mixed biofilms may reinforce bacteria pathogenicity.

In conclusion, we found that co-culture may result in increased antibiotic resistance and upregulated virulence gene expression for *S. suis* and *A. pleuropneumoniae* in biofilms. It is likely that the interspecies interactions between *S. suis* and *A. pleuropneumoniae* are synergetic under specific conditions. Therefore, the interactions between the species in the biofilm

community potentially influence the clinical course of disease. Our findings provide some relevant information that may affect the choice of antibiotics, and shed light on a new perspective for the treatment of mixed infection.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

AUTHOR CONTRIBUTIONS

YW and LY conceived and designed the experiments. SG, XD, and JL performed the experiments. YW, XD, and DG analyzed the data. LY and JL contributed reagents, materials, and analysis tools. YW and SG wrote the manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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