

Interaction Study of *Pasteurella Multocida* with Culturable Aerobic Bacteria Isolated from Porcine Respiratory Tracts using Coculture in Conditioned Media

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Abstract

Background: The porcine respiratory tract harbours various microorganisms, and the interactions between these organisms could be associated with animal health status. *Pasteurella multocida* is a culturable facultative anaerobic bacterium isolated from healthy and diseased porcine respiratory tracts. The interaction between *P. multocida* and other aerobic commensal bacteria in the porcine respiratory tract is not well understood. This study aimed to determine the interactions between porcine *P. multocida* capsular serotype A and D strains and other culturable aerobic bacteria isolated from porcine respiratory tracts using a coculture assay in conditioned media followed by calculation of the growth rates and interaction parameters.

Results: One hundred and sixteen bacterial isolates from five porcine respiratory tracts and 93 isolates were phylogenetically classified into fourteen genera based on 16S rRNA sequences. Fifteen representative isolates were selected for coculture with *P. multocida*. From 17x17 interaction pairs, the majority of 220 pairs had negative interactions indicating competition for nutrients and space, while 17 pairs were identified as mild cooperative or positive interactions indicating their coexistence. All conditioned media, except those of *Acinetobacter*, could inhibit *P. multocida* growth. Conversely, the conditioned media of *P. multocida* also inhibited the growth of nine isolates plus themselves.

Conclusion: Healthy porcine provides conditions to maintain the balance of these aerobic commensal communities and has an inhibitory effect on pathogens. Therefore, the conditioned media in this study might be further analysed to identify critical molecules. The study proposed the possibility of using these molecules in conditioned media to control *P. multocida* diseases and enhance porcine health.

Background

The porcine respiratory system is exposed to external environments and foreign particles, including bacteria, viruses and pollutants, through inhalation and exhalation processes [1, 2]. Respiratory diseases are associated with economic loss in the swine industry [3, 4]. Several bacteria predominantly colonize the porcine respiratory tract, including those in the phyla Firmicutes, Proteobacteria, and Bacteroidetes, and changes in these bacteria are often associated with porcine health status [5–8]. During porcine respiratory diseases, Proteobacteria in the family *Pasteurellaceae* and Bacteroidetes have been shown to abundantly increase, while the diversity of Firmicutes was shown to decrease [5, 7]. A member of the *Pasteurellaceae* family, *Pasteurella multocida*, commonly inhabits the nasopharynx of birds and mammals and can be associated with economically significant diseases, including fowl cholera, haemorrhagic septicemia in ungulates, progressive atrophic rhinitis (PAR) in swine, purulent rhinitis or snuffles in rabbits, enzootic pneumonia and shipping fever in sheep, cattle, and swine, and opportunistic infections in humans [9]. *P. multocida* has been classified into five capsular types (A, B, D, E and F) [10] and sixteen somatic or lipopolysaccharide (LPS) types [11] using serological methods.

P. multocida have been isolated from the nose, tonsils and upper respiratory tract of both healthy and diseased pigs [12–15]. The porcine toxigenic capsular type A and D strains of *P. multocida* can be primary pathogens or coinfect piglets with *Bordetella bronchiseptica*, causing PAR under stress and immunocompromised conditions [16]. Colonization of these strains in porcine tracheal rings increased during coinfection with *B. bronchiseptica* [17]. The adherence study of *P. multocida* and *B. bronchiseptica* to swine nasal epithelial cells found that *P. multocida* could not colonize the swine nasal mucosa well compared to *B. bronchiseptica*. The number of *B. bronchiseptica* cells adhered to the nasal epithelial cells was three times higher than the number of *P. multocida* cells, suggesting the opportunistic role of *P. multocida* after *B. bronchiseptica* infection [18]. A toxigenic *P. multocida* strain could assist the colonization of *B. bronchiseptica* [19, 20]. The number of toxigenic strains correlated with the atrophic degree of the porcine nasal turbinates. The nontoxigenic capsular type A strains could be the primary agent of pneumonia and septicemia in 100-day-old pigs and caused dermatitis and nephropathy syndrome (PNDS) in growing and finishing pigs [12, 21]. Non-toxigenic *P. multocida* strains are also involved in porcine respiratory disease complex (PRDC) in association with other bacterial and viral pathogens [22]. For example, coinfection between *P. multocida* and *Mycoplasma hyopneumoniae* in pigs showed more severe clinical signs than single infection with *M. hyopneumoniae*, while infection with only *P. multocida* did not show clinical signs [18, 23]. Pseudorabies virus (PRV) coinfecting with *P. multocida* in porcine lungs showed severe lesions [24]. Coinfections with swine influenza virus (SIV), porcine respiratory syndrome virus (PRSV), and other primary pathogens have also been reported [25, 26]. The coinfection of *P. multocida* with other pathogens could enhance disease damage to the hosts; e.g., promote secure attachment of the bacteria to the bovine respiratory syncytial virus-infected cells [27] and increase inflammatory cells in the coinfecting lesions of bronchopneumonia pigs [28]. Although the interactions between *P. multocida* and other pathogens have been well-described, the interactions with commensal bacteria in the respiratory tract are less understood.

Microbiome analysis of porcine lungs using 16S rRNA and shotgun metagenomic sequencing found different bacterial communities between healthy and diseased lungs [6–8]. The majority of the bacteria in the pneumonic lungs were from the families *Mycoplasmataceae*, *Flavobacteriaceae* and *Pasteurellaceae*, including *Mycoplasma*, *Ureaplasma*, *Weeksella*, and *Pasteurella*, while more diverse bacterial families were in the *Mycoplasma hyopneumoniae*-carrying lungs [6]. The families *Mycoplasmataceae*, *Bradyrhizobiaceae* and *Flavobacteriaceae* were found to be common in carrier pigs. Huang *et al.* [8] used 16S rRNA metagenomic sequencing to examine 20 swine lungs. They found that the healthy lungs prevalently had bacteria from the genera *Methylobacterium*, *Prevotella*, *Sphingobium*, and *Lactobacillus*, whereas the genera *Mycoplasma*, *Ureaplasma*, *Sphingobium*, *Haemophilus*, and *Phyllobacterium* were abundant in the severe-lesion lungs. The microbial diversity inside these lesion lungs decreased when the population of certain bacteria increased. In piglets, *Streptococcus*, *Lactobacillus*, and *Actinobacillus* were the core bacterial genera in healthy piglets, while *Moraxella*, *Veillonella*, and *Porphyromonas* were higher in piglets with porcine respiratory disease [7]. Understanding the roles of the microbiome within the healthy respiratory tract could promote pig welfare by assisting nutrient absorption, biosynthesis of vitamins, metabolism of xenobiotics, and immune regulation; for example, the prevalence of

Lactobacillus in healthy porcine lungs compared with severe-lesion lungs suggests a potential protective role of these common bacteria [7, 8] and the reduction in Glässer's disease due to the occurrence of *Bacteroides* and the high level of bacterial species richness and diversity [5]. However, the bacterial interactions within the normal flora communities inside porcine respiratory tracts have not been entirely understood by microbiome studies. Coculture assays are frequently employed to study host-pathogen interactions [29]. For example, De Vos *et al.* [30] examined the polymicrobial interaction of 72 bacterial samples isolated from 23 individuals with urinary tract infections by using a coculture assay in spent media. The study found that competitive (-/-) and cooperative (+/+) interactions were more common than exploitive interactions (+/-) and that competitive interactions were enriched among individuals. As the interactions between *P. multocida* and other commensal bacteria in the porcine respiratory tract remain not well understood, this study aimed to initially determine the interactions between the porcine capsular type A and D strains of *P. multocida* and other culturable aerobic bacteria isolated from porcine respiratory tracts using a coculture assay in conditioned media. Understanding these interactions would benefit respiratory disease control to improve porcine health and welfare.

Results

1. Culturable aerobic bacteria from porcine respiratory tracts

One hundred and sixteen aerobic bacterial isolates from five porcine respiratory tracts were successfully cultured from the trachea, tracheobronchial lymph node, epical lobe, cardiac lobe, and diaphragmatic lobe of both the left and right lungs. An average of 23 ± 10 isolates was obtained from each lung. The L1 and L4 lungs had the highest numbers of 31 and 36 isolates, respectively. The majority of the isolates (56%) were from the epical and diaphragmatic lobes of the lungs. Almost 90% of these isolates were gram-negative rod-shaped bacteria that had different colony characteristics, i.e., colony forms (63% circular and 37% irregular), margins (55% undulate, 26% entire, 18% curled, and 1% lobate), and mucosity (66% nonmucoid and 34% mucoid). These culturable isolates represented a proportion of aerobic commensal bacteria inside the porcine respiratory tracts in this study. Ninety-three (80%) of these aerobic culturable bacteria were successfully identified and classified into 14 genera and 21 species (Additional file 1) from seven families of three bacterial phyla (97% Proteobacteria, 17% Firmicutes, and 2% Bacteroidetes), i.e., *Acidovorax*, *Acinetobacter*, *Aeromonas*, *Escherichia*, *Enterobacter*, *Hafnia*, *Klebsiella*, *Macrococcus*, *Proteus*, *Providencia*, *Shewanella*, *Shigella*, *Weeksella*, and *Wohlfahrtiimonas*, based on 16S rRNA sequence analysis (Figs. 1 and 2). Phylogenetic analysis clustered these 93 isolates into seven major groups (Fig. 2). The first four groups (60%) were members of the family *Enterobacteriaceae*, including *Escherichia*, *Shigella*, *Enterobacter*, *Klebsiella*, *Hafnia*, *Proteus*, and a small cluster of *Providencia*. The fifth group contained *Macrococcus*, which was the only gram-positive bacterial genus belonging to the family *Staphylococcaceae*. The sixth and seventh groups consisted of the genera *Acinetobacter* (family *Moraxellaceae*) and *Aeromonas* (family *Aeromonadaceae*). The remaining isolates had only one or two members, including *Acidovorax* (family *Comamonadaceae*), *Shewanella* (family *Shewanellaceae*), *Wohlfahrtiimonas* (unclassified bacteria in the class Gammaproteobacteria), and *Weeksella* (family *Flavobacteriaceae*). One isolate was selected to represent each identified genus, except two isolates of

Macrococcus, NS20 in the fifth group containing *Macrococcus* samples and NS108 in the seventh group together with *Aeromonas*.

2. Growth of the selected aerobic bacterial isolates from the porcine respiratory tracts in different conditioned media

Fifteen isolates from 14 genera of the isolated aerobic bacteria from the porcine respiratory tracts and two porcine strains of *P. multocida* with capsular types A and D (PM7 and PM2) were cocultured in the conditioned media (spent BHIB) and the unconditioned media (fresh BHIB), resulting in 289 interacting pairs (17 × 17) as shown in Fig. 3. Nearly all conditioned media could inhibit the growth of these two *P. multocida* strains, except that of *Acinetobacter*. The conditioned medium of *Acinetobacter* supported or slightly slowed the growth of all tested bacteria. The conditioned medium of *Providencia* inhibited the growth of every isolate, including itself. The media of *Shigella* and *Macrococcus* Group 7 (G7) had a lower inhibitory effect on *Klebsiella*, *Escherichia*, *Shigella*, and *Enterobacter*. Conditioned media from *Proteus* and *Escherichia* only supported the growth of *Klebsiella* with a prolonged lag phase. The media of five bacterial samples (*Klebsiella*, *Shewanella*, *Acidovorax*, *Enterobacter*, and *Hafnia*) only inhibited the growth of *P. multocida*. The media of both *P. multocida* strains similarly inhibited *Aeromonas*, *Wohlfahrtiimonas*, *Shewanella*, *Acidovorax*, *Macrococcus*, *Acinetobacter*, *Providencia*, and *Weeksella* as well as themselves. The media of the remaining four samples (*Weeksella*, *Wohlfahrtiimonas*, *Aeromonas*, and *Macrococcus* G5) had different effects on the tested bacteria. Some conditioned media could promote bacterial growth compared to the control. For example, *Weeksella* grew better in the conditioned media of five bacterial samples (*Acinetobacter*, *Wohlfahrtiimonas*, *Shewanella*, *Acidovorax*, and *Enterobacter*).

3. Interaction between the porcine strains of *Pasteurella multocida* and the selected aerobic bacteria from the porcine respiratory tracts

This study measured bacterial interactions using the interaction parameter ϵ , which was calculated from the log ratio of maximum growth yield in the conditioned medium compared with that in the unconditioned medium. Pairwise interactions between 17 bacterial isolates revealed that most of the interactions (220 interactions) were negative interactions (Fig. 4). All negative interactions (-/-) were observed when growing the isolates in the conditioned media from *Escherichia*, *Macrococcus*, *Pasteurella*, *Proteus*, *Providencia*, *Shigella*, and *Weeksella*. Strong negative interactions (59 interactions, $\epsilon < -1$) were observed in the conditioned media of *Providencia* (17 interactions), *Macrococcus* G5 (12 interactions), *Escherichia* (11 interactions), *Shigella* (11 interactions), and *Weeksella* (3 interactions), and four interactions were observed in the media of *Aeromonas*, *Klebsiella*, and *Wohlfahrtiimonas*. Most of these spent media, except that of *Wohlfahrtiimonas*, had a pH between 5.4–6.5, which was lower than the pH of the reference medium BHIB (7.4) (top dendrogram in Fig. 4). Conditioned media from four bacteria (*Aeromonas*, *Klebsiella*, *Macrococcus* G5, and *Providencia*) had a strong negative effect on *P. multocida* growth. Notably, the medium of *Providencia* (pH 5.5) had a strong negative interaction with all tested isolates, including itself. The low pH (5.4) of the media from the two *P. multocida* strains resulted

in mild to moderate negative interactions with the other tested bacteria. The interaction patterns of *P. multocida* with these 17 conditioned media were separated from those of other bacterial samples (as shown in the right dendrogram) similar to the second cluster of four isolates from the Enterobacteriaceae family and the third cluster of ten samples. Seventeen mild positive interactions (+/+, $0 < \varepsilon < 0.1$) were observed with the media of *Acidovorax*, *Acinetobacter*, *Aeromonas*, *Enterobacter*, *Hafnia*, *Klebsiella*, *Shewanella*, and *Wohlfahrtiimonas*. Six of these interactions observed in spent media (*Acidovorax*, *Acinetobacter*, *Enterobacter*, *Hafnia*, *Shewanella*, and *Wohlfahrtiimonas*) had pH values between 6.5–7.3, which were close to the pH of BHIB. *Weeksella* had positive interactions in five conditioned media, *Acidovorax*, *Acinetobacter*, *Enterobacter*, *Shewanella*, and *Wohlfahrtiimonas*, which was the highest number among the media (highlighted in red in Fig. 4). The conditioned medium of *Enterobacter* supported the highest number of positive interactions with five bacteria, which included *Acinetobacter*, *Acidovorax*, *Macrococcus* G7, *Wohlfahrtiimonas*, and *Weeksella*. A high proportion of mild negative interactions (88 interactions, $0 > \varepsilon > -0.1$) was observed from bacteria grown in nearly all media, except *Providencia* and *Shigella*. These mild positive and negative interactions could be classified as neutral interactions. However, strong positive interactions were not observed in this study.

By focusing on the locations of the porcine respiratory tract in Fig. 5, the trachea (T) had the least number of bacterial genera (5) and had *Providencia* and *Shigella* as strong negative influencers. *Macrococcus* G7 and *Shigella* had substantial negative impacts on the others in the tracheobronchial lymph node (TN), while *Acidovorax*, *Acinetobacter*, *Enterobacter*, and *Klebsiella* provided positive support to some bacteria in this community. For the epical, cardiac, and diaphragmatic lobes of the porcine lung, *Macrococcus* G7 was the major bacteria that had a strong negative interaction with the others, except for the epical lobe, in which *Providencia* also exerted a negative effect. These three lobes of the lung shared six common bacteria with mild negative or positive interactions (*Acinetobacter*, *Escherichia*, *Enterobacter*, *Klebsiella*, *Macrococcus* G5, and *Proteus*), whereas *Shewanella* and *Wohlfahrtiimonas* were unique to the epical lobe, *Hafnia* was unique to the cardiac lobe and *Weeksella* was unique to the diaphragmatic lobe.

Discussion

The porcine respiratory tract has a large mucosal surface area suitable for the colonization of several bacteria, including pathogens [31]. Our study focused on bacterial isolates from porcine respiratory tracts that were culturable and able to grow under aerobic conditions due to the ease of culture and handling so that the isolates could be used for initial coculture experiments to examine their interactions with opportunistic/pathogenic bacteria such as *P. multocida*. Future utilization and application of these aerobic bacterial isolates from farms would not require extensive preparations and conditions. Some metagenomic studies in the porcine respiratory tracts have been conducted, and different groups of abundant bacteria between healthy and diseased pigs were found [5–8]. From 116 isolates, our study identified 14 bacterial genera, which included four aerobic (*Acidovorax*, *Acinetobacter*, *Weeksella*, and *Wohlfahrtiimonas*) and ten facultative anaerobic (*Aeromonas*, *Escherichia*, *Enterobacter*, *Hafnia*, *Klebsiella*, *Macrococcus*, *Proteus*, *Providencia*, *Shewanella*, and *Shigella*) bacterial genera belonging to seven families (*Aeromonadaceae*, *Comamonadaceae*, *Enterobacteriaceae*, *Flavobacteriaceae*,

Moraxellaceae, *Shewanellaceae*, and *Staphylococcaceae*) under three phyla (Proteobacteria, Firmicutes, and Bacteroidetes) from the respiratory tracts of healthy pigs. Bacteria in the genus *Macrococcus* (family *Staphylococcaceae*) were the only gram-positive bacterial group isolated in this study. These bacteria are normal flora commonly found in pigs by nasal swabs and in food products and environments. The 16S rRNA sequence analysis in the current study showed two distinct isolates of *Macrococcus caseolyticus* (G5 and G7) with different patterns of negative interaction despite their similar colony and cell morphology. The 16S rRNA sequencing of *M. caseolyticus* G5 was repeated twice, and the results confirmed the placement of this isolate in the clade of *Aeromonas* (99% sequence identity). In comparison, the 16S rRNA sequences of *M. caseolyticus* G7 showed 76% sequence identity to that of *Aeromonas*, suggesting a precaution of interpreting this sequence for species identification or further comparative characterization of the two isolates.

Some of these 14 bacterial genera were previously reported in porcine respiratory tract metagenomic studies [5–7, 32]. Proteobacteria, Firmicutes, and Bacteroidetes were also found to be abundant in studies of the nasal and oropharyngeal microbiota of piglets. The identification of *Acinetobacter*, *Klebsiella*, and *Weeksella* in this study was consistent with the abundant bacteria observed within the nasal microbiota of healthy piglets from farms in the UK and Spain [5]. *Klebsiella* and *Weeksella* were also reported as members of the core nasal microbiota. *Weeksella* could be isolated in certain parts (tracheobronchial lymph node and the diaphragmatic lobe of the lung) of the respiratory tracts in this study, suggesting a change in microbiota from the nasal cavity to the lungs. When comparing our results performed in mature pigs to the results of the study of oropharyngeal microbiota from healthy piglets in China [7], only *Escherichia* and *Shigella* were shared, which showed high abundance in piglets with respiratory diseases. *Streptococcus*, *Lactobacillus*, and *Actinobacillus* were the core microbiota in the oropharynx of these healthy piglets. In contrast, our study found six bacteria (*Acinetobacter*, *Escherichia*, *Enterobacter*, *Klebsiella*, *Macrococcus*, and *Proteus*) to be the core aerobic microbiota inside the lungs, which was also different from a metagenomic study of the microbiota inside the lungs of healthy pigs in Brazil [6]. Their common microbiota included the families *Mycoplasmataceae*, *Bradyrhizobiaceae*, and *Flavobacteriaceae*, whereas only *Aeromonas*, *Escherichia*, and *Weeksella* were shared with the present study. The microbiota of sac and gland-like tissues might be maintained, and the changes may be limited better than the hollow tract with the mucosal surface exposed to the air space within the respiratory tracts. For example, Lowe *et al.* [33] studied the microbial community inside the tonsils of healthy pigs using culture-dependent and culture-independent methods by sequencing 16S rRNA clone libraries. They were able to identify common bacteria (*Actinobacillus*, *Enterobacter*, *Klebsiella*, *Pasteurella*, *Proteus*, and *Providencia*) from porcine tonsils by both methods, and the results were similar to our result. These results suggested that the internal parameters of the pigs (age and tissue types) and environmental factors could affect the change in microbiota. Although the bacteria isolated in our study could represent a subset of the porcine respiratory tract microbiota, the findings began to reveal interactions of the normal flora community within the porcine respiratory tract, and further examination of the remaining community is required.

Opportunistic bacteria, e.g., *Pasteurella*, *Haemophilus*, and *Actinobacillus*, were also abundant in the nasal, oropharynx, and tonsil of healthy pigs but were not isolated under aerobic conditions in this study, which seemed to be a limitation of the method compared to the metagenomics approach [5, 7, 34]. Certain gram-negative bacterial isolates (*Providencia*, *Shigella*, *Escherichia*, and *Proteus*) in this study yielded conditioned media that strongly inhibited the growth of other bacteria. As they could cause opportunistic infections in animals, this suggested possible chemical-related mechanisms of these bacteria to imbalance the normal flora community in the porcine respiratory tract. Moreover, our coculture assay showed that conditioned media from aerobic bacterial isolates from 13 genera could inhibit the growth of *P. multocida*. The counteract phenomenon had never been reported and suggested that oxygen availability to aerobic bacteria might be necessary for the control of anaerobic or facultative anaerobic pathogens in the mucosal layer of the porcine respiratory tracts. A thickened mucosal layer during respiratory disease progression could favour the growth of facultative anaerobic or anaerobic pathogens, whereas limited oxygen accessibility might weaken aerobic pathogens and perhaps favour disease development.

Although our results covered a subset of the bacterial community in porcine respiratory tracts as determined by metagenomics studies, the study successfully narrowed and selected particular groups of aerobically grown bacteria from the diverse community and shed light on their interactions with *P. multocida*, which might not be easily assessed by whole genome shotgun metagenomics. Most bacterial interactions in this study were negative interactions, which represented the competitive need of these bacteria to share resources and spaces within the host [35]. During the preparation of the conditioned media, bacteria multiplied and spent nutrients in the media, so the conditioned media would have fewer nutrients and plenty of metabolites. Normal growth of bacteria in the conditioned media might imply that these bacteria could co-inhabit the same area (positive and mild negative interactions), while those affected by scarce nutrients, metabolites, and unsuitable pH would not be able to thrive together. Bacterial competition also involves several molecular mechanisms. For example, *Streptomyces* could inhibit antibiotic production by other bacterial competitors to increase its antibiotic production [36]. Barger *et al.* [37] found that *Streptomyces* secreted a combination of metabolites and enzymes to degrade colonies and cause cellular lysis of *Bacillus subtilis*. Competition could cause disproportional populations in the bacterial community and may alter functional relationships in that ecosystem [38]. These interactions could also change the growth conditions of bacteria in the community, increasing or decreasing community complexity [39]. Aside from the effect of bacterial secretion, the rise of one bacterial population could decrease resource availability for another species in the microbiome system. The results of this study also showed that *P. multocida* had negative interactions with several bacteria and that their conditioned media also inhibited the growth of many bacterial isolates. However, *P. multocida* could not begin its log phase in almost all conditioned media. The pathogens might have to compete and control the growth of the normal flora bacteria to initiate their multiplication. Competition could also occur within the same bacterial population as in the case of *Providencia*, suggesting a process to control the population size and initiate spreading to neighbouring areas. The condition that enhances the growth of the normal flora community would provide an inhibitory effect on the pathogens. Therefore,

the conditioned media in this study might be further characterized to identify key molecules that could control the bacterial population within the community. In addition to the negative interactions, mild positive interactions were detected for some pairs. Cooperation between microorganisms was reported in a study by Deng and Wang [40], who compared the growth, metabolic activity and enzyme production between pure and mixed cultures in glucose and lignocellulose media. They found that cooperation was common in the lignocellulose media, which promoted positive interactions and synergistic growth. Glucose media promoted negative interactions and competition between organisms in mixed cultures. A study by de Vos *et al.* [30] also showed that the interaction between bacteria in conditioned media increased bacterial tolerance to antibiotics, and positive interactions were observed under non-antibiotic conditions. By knowing this, we proposed a possibility to use the molecules in the conditioned media to control *P. multocida* outbreaks and enhance porcine health.

Conclusions

One hundred and sixteen bacterial isolates were collected from five porcine respiratory tracts, and 93 isolates were phylogenetically classified into fourteen genera based on 16S rRNA sequences. The coculture of 15 representative isolates and two strains of *P. multocida* showed a majority of negative interactions with a few cooperative/positive interactions. All conditioned media, except those of *Acinetobacter*, could inhibit *P. multocida* growth. Conversely, the conditioned media of *P. multocida* also inhibited the growth of eight isolates plus themselves. Thus, this study proposed the possibility of using the molecules in conditioned media to control *P. multocida* diseases and enhance porcine health.

Methods

1. Bacterial isolation from porcine respiratory tracts

Five porcine respiratory tracts were screened and collected from slaughterhouses in Nakhon Pathom and Ratchaburi provinces, Thailand, via the assistance of D.V.M. Pichai Joipang from B.F. Feed Co., Ltd. Bacterial samples were isolated from different parts of the respiratory tract, i.e., trachea, tracheobronchial lymph node, epical lobe, cardiac lobe and the diaphragmatic lobe of both the left and right lungs. The samples were spread on tryptose agar supplemented with 5% sheep blood and McConkey agar and then incubated aerobically overnight at 37 °C. Colony morphology was observed, and distinct colonies were selected for further subculture on tryptose blood agar. After incubating aerobically overnight at 37 °C, a single colony was picked and subcultured until the pure isolate was obtained. The pure isolate was smeared on a glass slide and checked for purity and bacterial cell morphology by Gram staining and microscopic observation. Extraction of genomic DNA from the pure isolates was performed using the GF-1 bacterial DNA extraction kit (Vivantis, Malaysia), and the genomic DNA was stored in 50% glycerol with brain and heart infusion broth (BHIB) at -80 °C until use.

2. Bacterial identification by 16S rRNA nucleotide sequencing

Bacterial genera and species were identified by PCR amplification of the 16S rRNA gene using SR-FWD (5'-AGAGTTTGATYMTGGC-3') and SR-REV (5'-GYTACCTTGTTACGACTT-3') as forward and reverse primers, respectively [41]. PCR was carried out in a final volume of 20 μ L containing 2 μ L of DNA template, 0.4 μ L of Taq DNA polymerase (Vivantis, Malaysia), 2 μ L for each of 2 μ M SR-FWD and SR-REV primers, 0.6 μ L of 50 mM MgCl₂, 2 μ L of 2 mM dNTPs (Vivantis, Malaysia), 2 μ L of 10X Buffer A (Vivantis, Malaysia) and 9 μ L of distilled water. PCRs were initially denatured at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 45 sec, annealing at 50 °C for 45 sec, extension at 72 °C for 1.35 min, and a final extension at 72 °C for 5 min using a thermal cycler (Bio-Rad Laboratory Inc., Germany). PCR products were quantified and checked for quality using a NanoDrop 2000 (Thermo Scientific, Germany) before separating on 1% agarose gel electrophoresis and visualizing under a UV transilluminator (Bio-Rad, United States). The PCR products were purified using the GF-1 AmbiClean kit (Vivantis, Malaysia) and subjected to Sanger nucleotide sequencing (Macrogen, Korea).

3. Selection of representative bacterial isolates by 16S rRNA sequence analysis and phylogenetic reconstruction

The obtained nucleotide sequences of the 16S rRNA gene were trimmed and merged by using the BioEdit program version 7.0.5.3 [42]. The sequences were searched against the NCBI nucleotide database using the blastn program [43] to identify closely similar bacterial genera and possibly the closest species. The identification was decided based on the Blast query score, e-value equal to 0, and percentage of sequence identity greater than or equal to 99%. As many bacterial isolates were examined and certain isolates belonged to the same genus and species, representative isolates of these bacteria were selected by the following steps. The 16S rDNA sequences of bacterial isolates belonging to the same genus were multiple aligned by using the clustalW algorithm in the BioEdit program version 7.0.5.3 [42, 44]. If the percentage of sequence identity was more than 95%, the sequences were classified into the same genera. If the identity was lower than 95%, the sequences were considered as different genera. The phylogenetic relationship of these 16S rDNA sequences was reconstructed from the aligned sequences based on the maximum likelihood algorithm and Jukes-Cantor substitution model with 1,000 bootstrap iterations using the phangorn package in R [45, 46]. The phylogenetic tree was visualized by the ggtree package in R [47]. The phylogenetic data were then combined with the colony and cell morphology, Gram staining pattern, and locations where they were isolated in the respiratory tracts. At least one isolate representing the same genus was chosen from the cluster and used to prepare conditioned media for the coculture assay.

4. Preparation of the conditioned media for the coculture assay

The conditioned medium was spent medium from the culture of a bacterial isolate. All selected bacterial isolates were revived on tryptose blood agar and incubated overnight at 37 °C before subculture into 40 mL of BHIB and incubation for 48 h at 37 °C and 180 rpm. Bacterial cells were pelleted by centrifugation at 4,800 $\times g$ at room temperature for 15 min. The supernatant medium was filtered by a 0.2 μ m polyethersulfone (PES) membrane filter (Whatman, United Kingdom) and a 50 mL syringe (Nipro,

United States). The pH of all conditioned media was measured using a pH meter (AZ Instrument Corp., Taiwan), and the conditioned media were stored at 4 °C until use.

5. Coculture assay and bacterial growth measurement

Two porcine strains of *P. multocida* (capsular types A and D isolated from pneumonia pigs in Thailand) and the selected isolates of culturable aerobic bacteria were revived on tryptose blood agar and incubated overnight at 37 °C before subculture into 1 mL of BHIB. The coculture assay began by adding 200 µL of the conditioned medium into the nontreated transparent flat-bottom 96-well plate followed by inoculating 0.2 µL of the overnight bacterial culture. Each bacterial isolate was grown in conditioned media from all chosen isolates, including itself. The coculture was incubated at 37 °C and 180 rpm for 40 h. The optical density at 600 nm (OD₆₀₀) was measured every hour using a microplate spectrophotometer (PowerWave 340, BioTek, United States), and each condition was performed in triplicate. The bacterial growth rate was calculated using the following logistic equation :

$$N_t = \frac{K}{1 + \left(\frac{K-N_0}{N_0} \right) e^{-rt}}$$

where N_t represents the population size at time t , and N_0 is the population size at the beginning of the growth curve. The maximum population size in the particular environment was limited to the carrying capacity parameter K . The OD₆₀₀ values from each condition were fit into this logistic equation to generate the growth curve model by using the SummarizeGrowth function of the Growthcurver package in R [48]. The growth curves were plotted and compared using the ggplot function of the ggplot2 package to visualize the effect of conditioned media on different bacterial isolates [49].

6. Determination of the bacterial interactions from the coculture assays

The definition of the bacterial interaction in this study was adjusted from the study of de Vos *et al.* [30]. The bacterial interaction was expressed as an interaction parameter ε , described in the following equation:

$$\varepsilon = \log(N_c/N_r)$$

where N_c is the growth yield in the conditioned medium, and N_r is the growth yield in the reference medium or fresh BHIB. The growth yield was defined by the average of the four highest OD₆₀₀ values from the growth curve. The mean of the triplicate maximum growth values in each condition was used to calculate the interaction parameter. A positive ε value ($\varepsilon > 0$) means that the growth yield in the conditioned medium was higher than that of the reference, indicating the positive or cooperative interaction (+/+) of the two bacterial isolates. A negative or competitive interaction (-/-) corresponds to a negative ε value ($\varepsilon < 0$). These parameters were then used to explain the interactions between *P. multocida* and the selected bacterial isolates.

Declarations

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Ethics approval and consent to participate

Not applicable.

Available data and material

The dataset supporting the conclusions of this article is included within the Additional file 1.

Consent for publication

Not applicable.

Competing interests

No competing interests.

Authors, contributions

Hanchanachai carried out bacterial isolation, identification and interaction studies and data analysis. Hanchanachai, Chumnanpuen, and E-kobon planned and designed the experiments as well as performed data analysis and discussion. Hanchanachai drafted the manuscript; E-kobon edited and revised the manuscript, and all authors read and approved the final manuscript.

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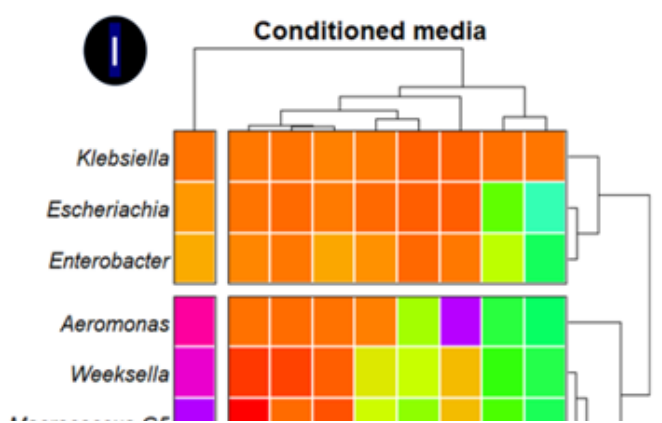
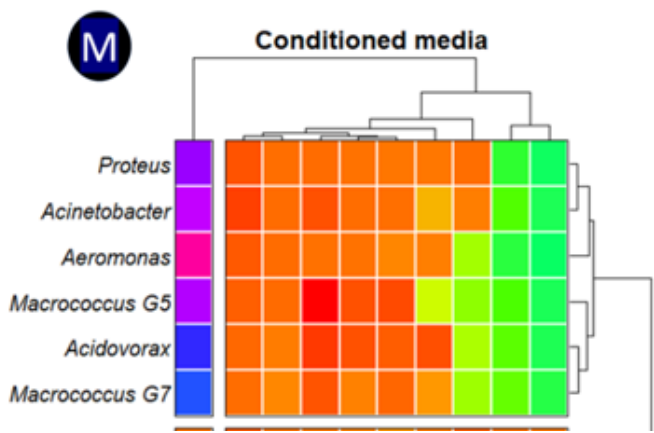
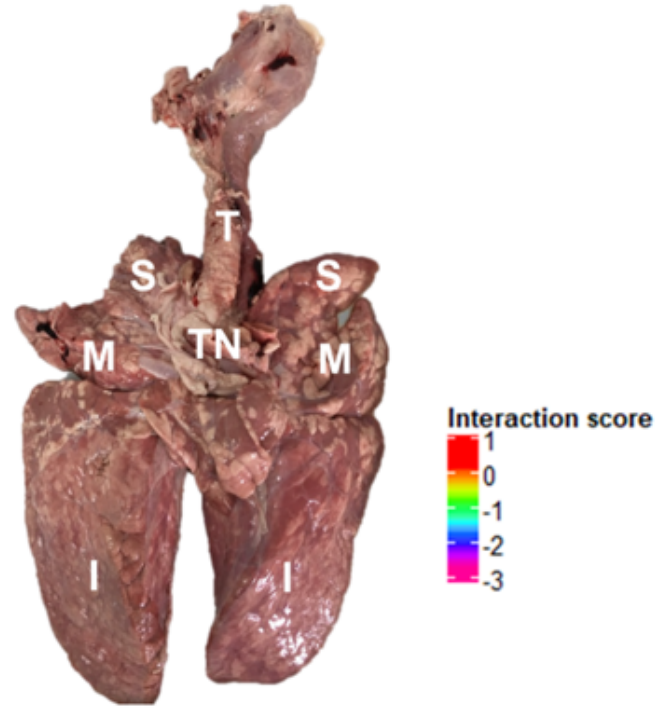
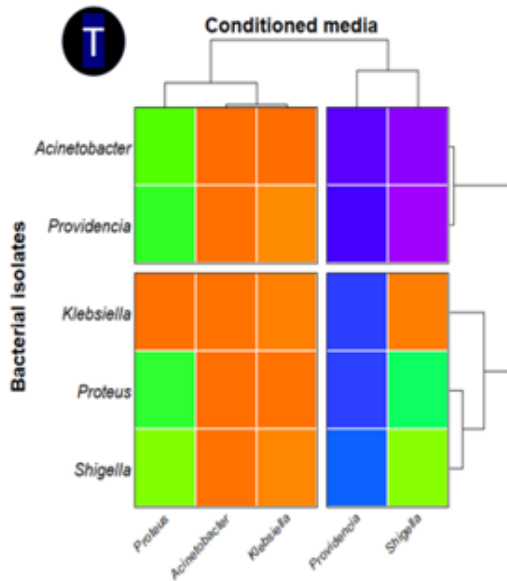
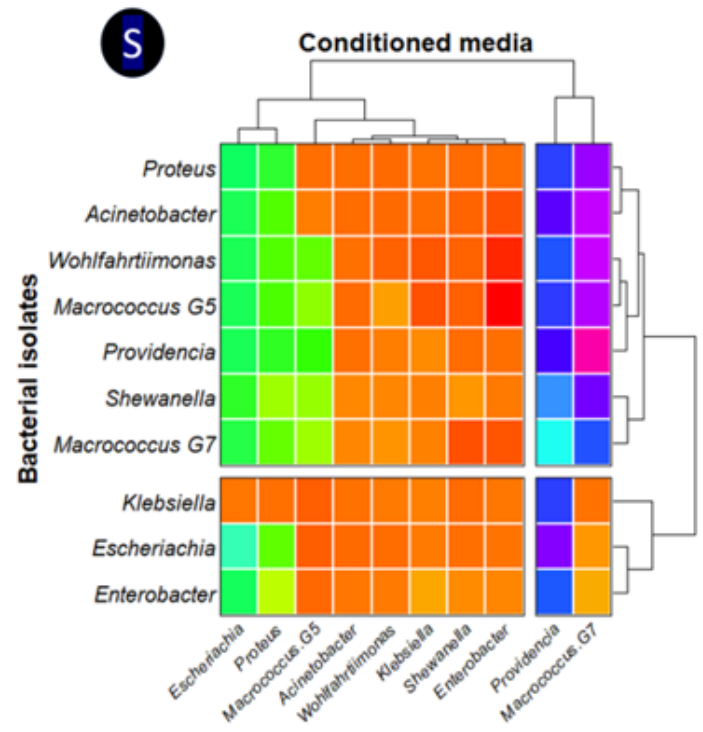
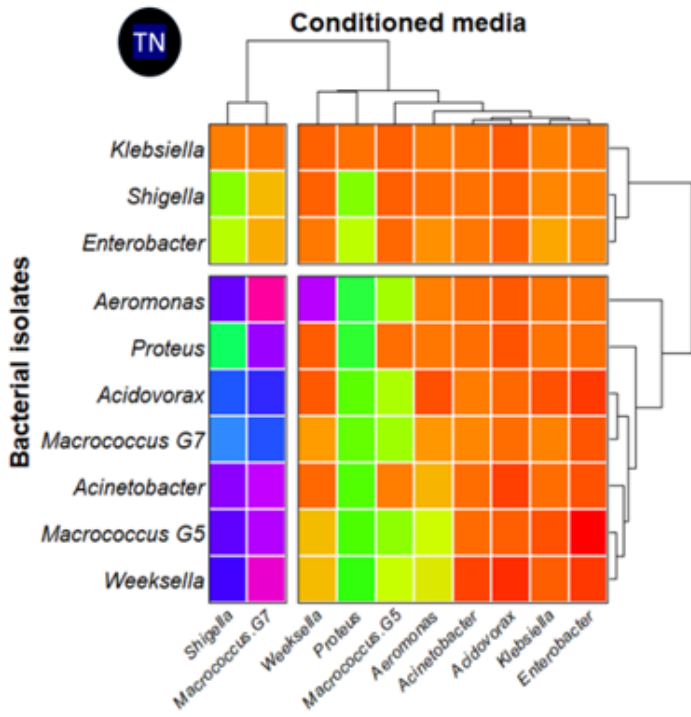
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Figures



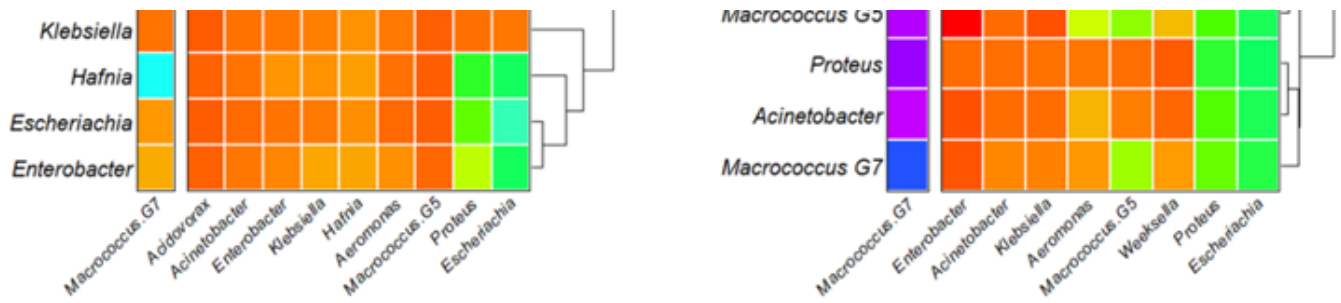


Figure 1

Bacterial interaction values (ϵ) clustered by locations of the porcine respiratory tracts. T, trachea; TN, tracheobronchial lymph node; S, epical lobe; M, cardiac lobe; and I, diaphragmatic lobe of the left and right lungs. A positive interaction score represented a positive interaction (+/+), and a negative interaction score represented a negative interaction (+/-). The top dendrogram shows the conditioned media clustering, and the right dendrogram shows the genera clustering.

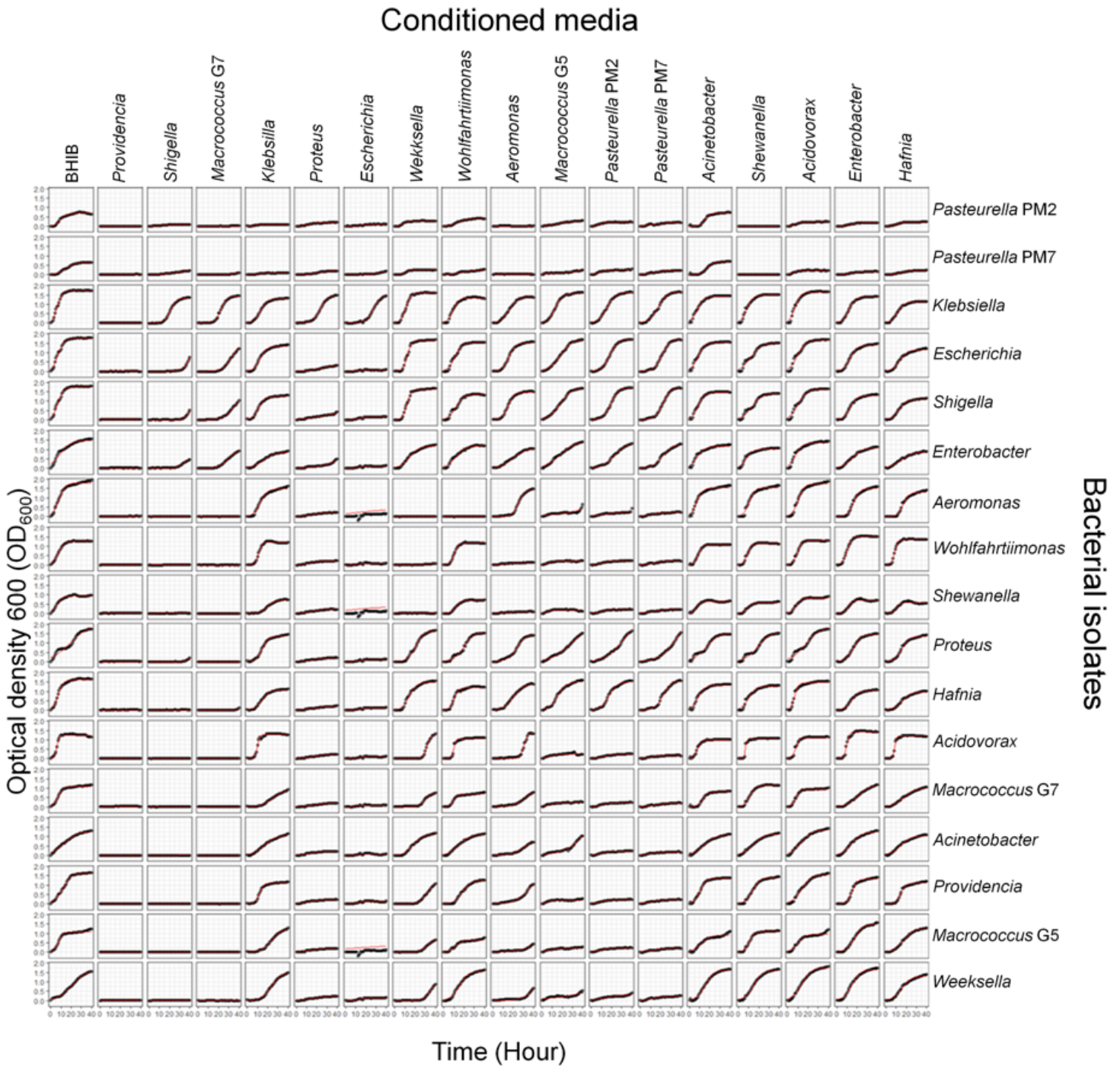


Figure 3

Comparative growth rate of 15 isolates (14 genera) of aerobic bacteria from the porcine respiratory tract and two porcine strains (PM2 and PM7) of *P. multocida* grown in different conditioned media compared to growth in complete medium (BHIB). The optical density at 600 nm was measured hourly for 40 h, and the growth rate was calculated using the logistic equation.

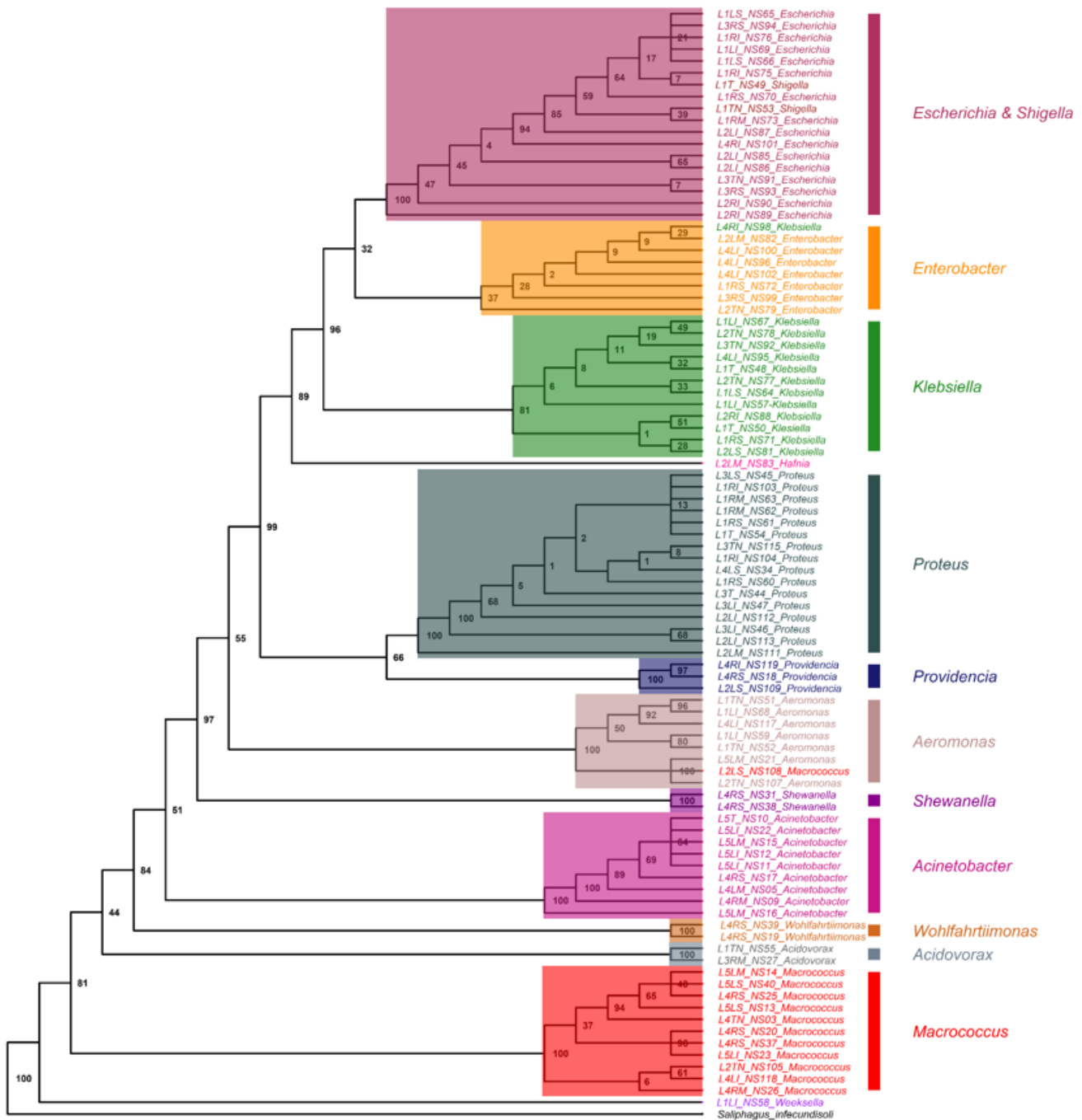


Figure 4

Phylogenetic relationships of the 16S rRNA gene from 93 culturable aerobic bacterial isolates from porcine respiratory tracts constructed by the maximum likelihood model with 1000 bootstraps (shown as percentage numbers at the node of the tree) and using the sequence of *Salphagus infecundisoli* as an outgroup. Seven major clusters were highlighted with different coloured boxes, and the genera were labelled with different colours on the right of the tree.

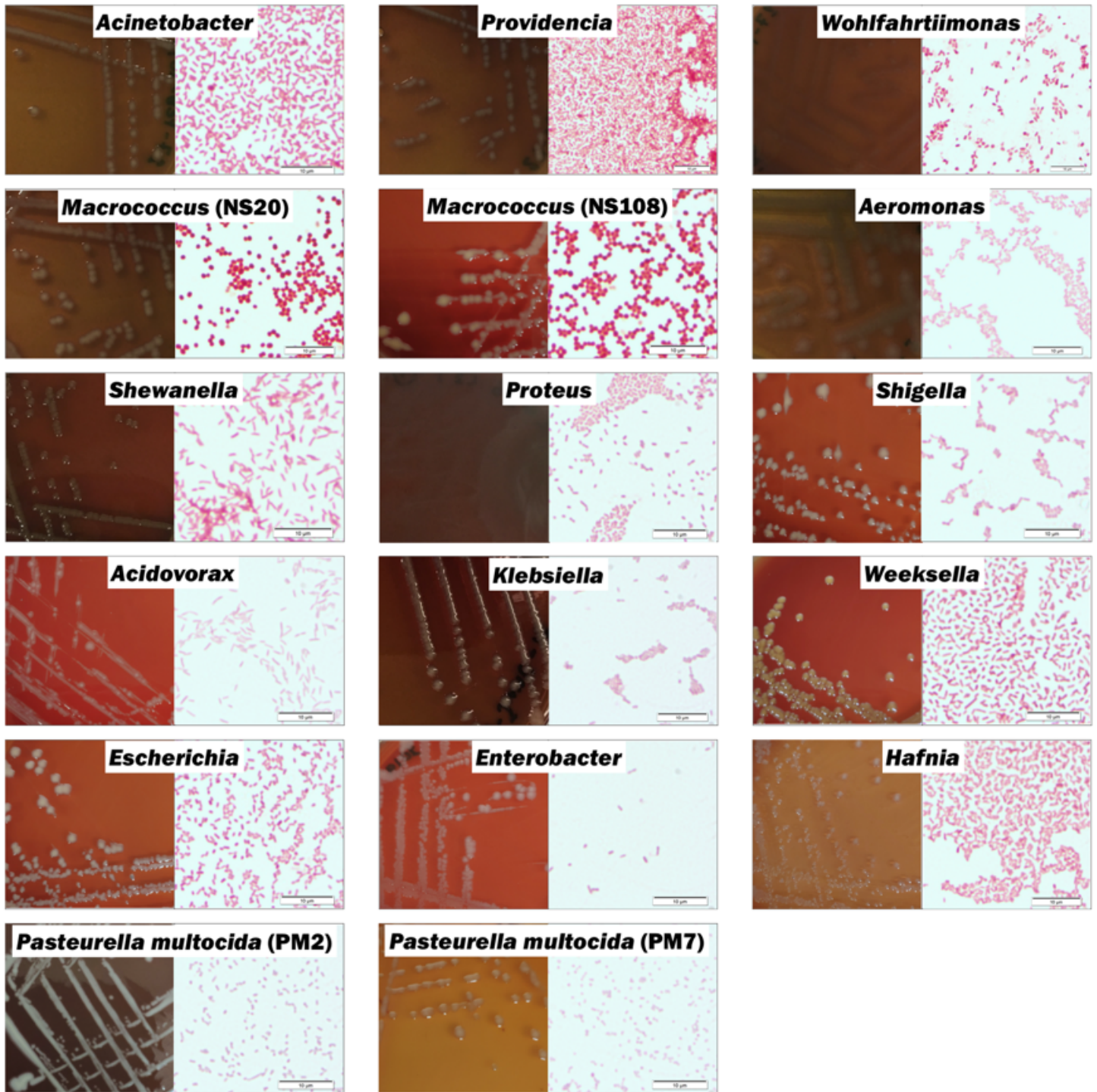


Figure 5

Colony and cell morphology of 15 culturable aerobic bacterial isolates obtained from porcine respiratory tracts and chosen for the coculture assay in conditioned media with two porcine strains (PM2 and PM7) of *P. multocida*. Colony morphology was observed from the growth on agar plates, and the Gram-stained cells were visualized under the microscope.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Additionalfile1Bacteriaisolatesdetailv2.xls](#)