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Doctoral Thesis

**Assessment and Mitigation of  
Airborne Contaminants from Swine  
Confinement Buildings**

February 2016

Graduate School of Seoul National University

Department of Agricultural Biotechnology

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**Assessment and Mitigation of  
Airborne Contaminants from Swine  
Confinement Buildings**

by

**Priyanka Kumari**

under the supervision of

**Professor Hong Lim Choi, Ph.D.**

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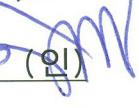
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# **ABSTRACT**

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The dominant factors influencing the abundance and community composition of bioaerosols in swine confinement buildings (SCBs) and their mitigation have been poorly studied. In this study, the indoor bioaerosols community structure and diversity were investigated in SCBs by using next generation sequencing platforms (454-pyrosequencing and Illumina). The effects of manure removal system and seasonal variations were investigated on bioaerosol communities obtained through NGS platforms. In this study, a biofiltration system was also used to explore how and to which extent it helped in mitigation of airborne contaminants emissions from SCBs.

The effect of manure removal systems (deep-pit manure removal with slats, scraper removal system, and deep-litter bed system) was studied on abundance and composition of airborne biotic contaminants in SCBs using cultivation-independent methods. The abundances of 16S rRNA genes and six tetracycline resistance genes (tetB, tetH, tetZ, tetO, tetQ, and tetW) were quantified using real-time PCR. The abundance of 16S rRNA gene and tetracycline resistance genes were significantly higher in SCBs equipped with a deep-pit manure removal system with slats, except for tetB gene. This observation contrasts with the trend found previously by culture-based

studies. The aerial bacterial community composition, as measured by pairwise Bray–Curtis distances, varied significantly according to the manure removal system. 16S rRNA-based pyrosequencing revealed *Firmicutes* (72.4 %) as the dominant group with *Lactobacillus* as the major genus, while *Actinobacteria* constituted 10.7 % of the detectable bacteria. *Firmicutes* were more abundant in SCBs with deep-pit with slats, whereas *Actinobacteria* were highly abundant in SCBs with a deep-litter bed system. Overall, the results of this study suggested that the manure removal system played a key role in structuring the abundance and composition of airborne biotic contaminants in SCBs.

Little is known about the seasonal dynamics of biotic contaminants in SCBs. The biotic contaminants of seven SCBs were monitored during one visit in winter and one during summer. Paired-end Illumina sequencing of the 16S rRNA gene, V3 region, was used to examine seasonal shifts in bacterial community composition and diversity. The abundances of 16S rRNA genes and six tetracycline resistance genes (*tetB*, *tetH*, *tetZ*, *tetO*, *tetQ*, and *tetW*) were also quantified using real-time PCR. Bacterial abundances, community composition and diversity showed strong seasonal patterns defined by winter peaks in abundance and diversity. Microclimatic variables of SCBs, particularly air speed, PM<sub>2.5</sub> and total suspended particles (TSP) were found significantly correlated to abundances, community composition, and diversity of bacterial bioaerosols. Seasonal fluctuations were also observed for four tetracycline resistance genes, *tetH*, *tetO*, *tetQ*, and *tetW*. The frequency of occurrences of these resistance genes were significantly higher in samples collected during winter and was also significantly correlated with air speed, PM<sub>2.5</sub> and

TSP. Overall, the results indicate that biotic contaminants in SCBs exhibit seasonal trends, and these could be associated with the microclimatic variables of SCBs.

Seasonal variations in community composition and diversity of airborne fungi were also studied in SCBs. Aerosol samples were collected from seven commercial swine farms in winter and summer. The internal transcribed spacer region 1 (ITS 1) of the ribosomal genes was sequenced using paired-end Illumina sequencing. Similarly to bacteria, indoor airborne fungal community composition and diversity were influenced by seasonal variations. However, the alpha and beta diversities showed very different patterns from one another, whereby alpha diversity peaked in winter and beta diversity peaked in summer. Several human allergen/pathogen related fungal genera were also identified in SCBs. Among these human allergen/pathogen related fungal genera, *Candida*, *Aspergillus*, *Pichia*, and *Trichosporon* varied significantly between seasons. In general, the relative abundance of human allergen/pathogen related fungal genera was higher in winter than in summer.

Biofiltration is known as one of cost-effective technology for treating the ventilation exhaust air from livestock buildings. However, little is known about the bacterial biofilm community immobilized in the packing material of biofilter which helps in breaking down of the contaminants present in air stream. A biological air filter system was used to reduce the emissions of airborne contaminants from SCBs, and also investigated the successional development of bacterial biofilm community in the packing material of biofilter by using the Illumina Miseq sequencing platform. It has been observed that the odorant

reduction efficiency of biofilter was increased linearly with time. The results also indicated that bacterial biofilm community structure of biofilter exhibited a strong time successional pattern, and to a lesser extent, filtration stage and the interaction between time and filtration stage also led to a significant variation in community structure of bacterial biofilm. Certain bacterial phyla and genera with ability to degrade various odorants got enriched at later time point of the experiment which might result in reduction of a wide variety of odorants.

Overall, it has been found that the indoor bioaerosol community composition and diversity are to a large extent structured by manure removal system and seasonal variations. It has been also observed that biofilter system efficiently reduced the emissions of a large number of odorous gases from SCBs, and the correlations established between bacterial biofilm community succession and odorants removal could be helpful in establishing better management strategies to minimize the potential health impacts on both the farm workers and the public residing in close proximity to these buildings.

**Keywords:** bioaerosols, biofilter, bacteria, fungi, Illumina, internal transcribed spacer, manure removal system, pyrosequencing, seasonal variations, swine confinement buildings, 16S rRNA gene.

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## **ABBREVIATIONS**

OTU: Operational taxonomic unit

PCR: Polymerase chain reaction

NGS: Next generation sequencing

SRA: Short read archive

rRNA: Ribosomal ribonucleic acid

NMDS: Non-metric multidimensional scaling

BLAST: Basic local alignment search tool

RDP: Ribosomal database project

DNA: Deoxyribonucleic acid

PERMANOVA: Permutational multivariate analysis of variance

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# **CHAPTER 1. Airborne Contaminants Present in Swine Confinement Buildings and Their Control: An Introduction**

## **1.2. Airborne contaminants present in swine confinement buildings**

Swine farming is revolutionized by the use of confined buildings all over the world. However, high animal densities in these confined environments lead to poor indoor air quality. The decomposition of animal feces and urine generates various gases (O'Neill & Phillips 1992), while feed and bedding materials, skin debris and dried manure produce particulate matter (PM) which helps in transportation of adsorbed microorganisms and endotoxins (Cabra-López *et al.* 2010). These airborne contaminants present in swine confinement buildings (SCBs) affects both animal and human health. Furthermore, the indoor air of SCBs is regularly ventilated to the outdoor environment, and the airborne contaminants present in the ventilated air can cause detrimental effects to the public residing in close proximity to these buildings. The airborne contaminants present in the SCBs are broadly categorized into three different types: particulate matter, gaseous compounds and bioaerosols. Particulate matter and gaseous compounds are discussed in following subsections, whereas bioaerosols are discussed in a separate section as most of the work in this study is on bioaerosols.

### **1.2.1. Particulate matter**

Particulate matter is a mixture of various types of pollutants with different physical, chemical and biological characteristics, and these characteristics determine the environmental and health impact of particulate matter (EPA 2004). The concentrations of PM are generally

10-100 times greater in livestock buildings compared to other indoor environments and PM carries various gases and microorganisms (Zhang 2004). Most of the PM present in SCBs are generated from feed materials (Donham *et al.* 1986; Heber *et al.* 1988; Honey & McQuitty 1979; Takai *et al.* 1998). The other possible sources of PM are skin debris and fecal materials of animals (Heber *et al.* 1988; Honey & McQuitty 1979). The concentrations of PM are also linked to swine activity (Kim *et al.* 2008b); with nursery swine houses have higher PM concentrations than finishing houses (Attwood *et al.* 1987). The main proportion of respirable PM has a diameter less than 5  $\mu\text{m}$  (Gustafsson 1999), and based on the site of deposition in body there are following types of PM (Carpenter 1986): PM > 10  $\mu\text{m}$ : nasal passage; PM 5-10  $\mu\text{m}$ : upper respiratory tract; PM < 5  $\mu\text{m}$ : lungs. It has been studied earlier in several studies that the respirable fraction of PM in SCBs varies from 7% to 13% (Donham 1986b; Gustafsson 1999). The presence of PM in SCBs can lead to severe respiratory diseases and loss of respiratory capacities in farm workers especially in young farm workers (Zejda *et al.* 1993). The exposure time to the indoor environment of SCBs can be associated to changes in respiratory capacities (Donham *et al.* 1989).

### **1.2.1. Gaseous compounds**

The most abundant gaseous compounds in the air of SCBs are ammonia ( $\text{NH}_3$ ), carbon dioxide ( $\text{CO}_2$ ), methane ( $\text{CH}_4$ ), hydrogen sulfide ( $\text{H}_2\text{S}$ ) and various volatile organic compounds (VOCs). The representative VOCs present in SCBs are sulfuric compounds, volatile fatty acids (VFAs), indolics and phenolics (Cai *et al.* 2006; Kai &

Schäfer 2004). Approximately 331 different VOCs and gaseous compounds have been reported in SCBs (Schiffman *et al.* 2001). These gaseous compounds are generated mainly due to microbial activity. In SCBs, the hydrolysis of urea led to generation of NH<sub>3</sub> from urine and swine slurry and this reaction is catalyzed by the enzyme urease (Mobley & Hausinger 1989). The level of dietary crude proteins influences NH<sub>3</sub> emissions as these are the primary source of nitrogen (Le *et al.* 2009), and it has been shown that NH<sub>3</sub> emissions can be limited by the reduction in dietary crude protein (Portejoie *et al.* 2004). Many other gaseous compounds (CH<sub>4</sub>, CO<sub>2</sub>, and H<sub>2</sub>S) are also produced by the microbial action on swine manure stored in manure pits under the building (Donham 1986a; Pedersen *et al.* 2008). Due to intensive animal stocking in confinement buildings, some gaseous compounds (NH<sub>3</sub> and H<sub>2</sub>S) can accumulate rapidly and become a respiratory hazard for both farm workers and animals. It has been also reported that the emission of the gaseous compounds to the outdoor environment are associated with mental health consequences, such as increased tension, depression, fatigue, confusion, and mood changes in members of surrounding communities (Schiffman *et al.* 1995; Schiffman & Williams 2005).

### **1.3. Bioaerosols and their characterization**

Bioaerosols comprises the biological particulates such as bacteria, fungi, viruses, endotoxin, and mycotoxin etc. suspended in air (Cox & Wathes 1995). Depending on the source, aerosolization mechanisms, and environmental conditions, bioaerosols can vary in

size (20 nm to >100 µm) and structure (Pillai & Ricke 2002). The inhalable fraction (1–10 µm) of bioaerosol is of primary concern because it can reach to deeper part of the respiratory tract (Stetzenbach *et al.* 2004). Both liquid and dry materials can act as source for bioaerosols. The usage of antibiotics in swine farms has promoted the development and abundance of antibiotic resistance in microbes (Blake *et al.* 2003; Zhu *et al.* 2013), which can become aerosolized within the SCBs. Antibiotic resistant genes (ARGs) can be transferred to pathogens through transformation or phage-mediated transduction, and/or by conjugation, posing a serious threat to public health. Pathogenic bioaerosols present in confinement buildings can cause direct harm to both farmworkers and animals. Bacterial pathogenic bioaerosols in SCBs can cause infectious and allergic diseases such as pneumonia, asthma, and rhinitis in workers and pigs (Donham *et al.* 1990; Pearson & Sharples 1995). The known allergenic, toxic, and inflammatory responses are caused by exposure not only to the viable but also to the non-viable microorganisms present in the air (Robbins *et al.* 2000). The presence of some pathogenic bioaerosols has been detected over long distances from their emission site which were found capable to infect healthy animals intramuscularly or intratracheally (Otake *et al.* 2010). It is clear that the swine confinement buildings are significant point sources of outdoor air contamination that can potentially pose a serious problem to public health.

### **1.2.1. Detection of bioaerosols by using cultivation-dependent approach**

Cultivation-dependent approach has been used initially to characterize the bioaerosols associated with SCBs. The predominant organisms cultured from bioaerosols of SCBs were bacteria and majority of the bacterial isolates from SCBs were Gram-positive species (Attwood *et al.* 1987; Chang *et al.* 2001; Crook *et al.* 1991; Donham *et al.* 1986; Mackiewicz 1998). The dominance of Gram-positive species in bioaerosols of SCBs suggested that swine feces are the primary source of the bacteria in the bioaerosols of SCBs, because 90% of the bacteria isolated from the feces of adult swine are reported as gram positive (Salanitro *et al.* 1977). The assessment of airborne bacteria in SCBs across the United States, Canada, The Netherlands, Sweden, Poland, and the United Kingdom showed that their concentration varied from  $10^5$  to  $10^7$  CFU/m<sup>3</sup> (Attwood *et al.* 1987; Clark *et al.* 1983; Cormier *et al.* 1990; Donham *et al.* 1989; Donham *et al.* 1986; Mackiewicz 1998; Nehme *et al.* 2008). It has been also found that the level of airborne bacterial count in SCBs showed seasonal variation with their concentrations peaking in summer season (Nehme *et al.* 2008).

The predominant bacterial genera identified in SCBs were *Aerococcus*, *Bacillus*, *Enterococcus*, *Micrococcus*, *Moraxella*, *Pseudomonas*, *Staphylococcus*, *Streptococcus* (Cormier *et al.* 1990; Crook *et al.* 1991; Predicala *et al.* 2002). The fungal colony forming units (cfu) reported in SCBs varied from  $10^3$  cfu/m<sup>3</sup> to  $10^6$  cfu/m<sup>3</sup> (Chang *et al.* 2001; Clark *et al.* 1983; Predicala *et al.* 2002). *Aspergillus*, *Cladosporium* and *Penicillium* were the most abundant fungal genera in SCBs. Several other fungal genera have been also detected in SCBs such as *Alternaria*, *Fusarium*, *Verticillium*, and

*Geotrichum*. Manure removal system in SCBs was shown to influence the indoor air fungal concentrations and emissions (Kim *et al.* 2008c). Keratinophilic (*Scopulariopsis brevicaulis*) and toxigenic fungi (*Aspergillus*, *Fusarium*, and *Penicillium* genera and *Stachybotrys chartarum*) were also detected on SCBs, suggesting a potential occupational health threat (Viegas *et al.* 2013). Jo *et al.* (2005) studied airborne fungi concentrations in swine sheds and reported that the summer concentrations of total fungi and fungal genera inside the swine sheds were substantially higher than the winter values. The initial characterization of bioaerosols based on cultivation-dependent techniques provided several important information about bioaerosols, however, cultivation-dependent techniques contain an inherent bias, as only the viable microbes that can be grown in culture are characterized. Furthermore, the majority of microorganisms cannot be cultured using standard cultivation techniques (DeLong & Pace 2001; Torsvik *et al.* 1996). This bias in cultivation led to overestimation of microorganisms that are easily cultured through standard cultivation techniques.

### **1.2.2. Detection of bioaerosols by using cultivation-independent metagenomic approach**

Cultivation-independent metagenomic approach bypasses the need of cultivation of microorganisms. Cultivation-independent metagenomic approach is mainly grouped into two categories; shotgun metagenomics and sequence-driven metagenomics based on their random and targeted sequencing strategies, respectively. There have been only few studies that have investigated the bioaerosol samples

collected from SCBs using cultivation-independent metagenomic approach. Nehme et al. (2009) examined bacterial and archaeal biodiversity using cultivation-independent metagenomic approach. Nehme et al. (2009; 2008) showed that the total bacterial concentrations in bioaerosol samples were 1,000 times higher than the concentration of airborne culturable bacteria. They also found that bacterial and archaeal concentrations in bioaerosol samples were significantly lower in summer than values obtained in winter, and bioaerosol microbial populations bear close resemblance to the fecal microbiota of confined pigs (Nehmé *et al.* 2009; Nehme *et al.* 2008). In another study, Kristiansen et al. (2012) also applied cultivation independent metagenomic approach to estimate the diversity and abundance of bioaerosolic bacteria and fungi in SCBs. They found more diverse bacterial and fungal community in bioaerosols of SCBs, and suggested that this could potentially create poor indoor air quality in SCBs. However, these used denaturing gradient gel electrophoresis and 16S-cloning-and-sequencing approach to characterize the microbial community in bioaerosol samples, which lack resolution and throughput, respectively, compared to next generation sequencing based methods (Bartram *et al.* 2011; Bent *et al.* 2007; MacLean *et al.* 2009).

The recent development of next-generation sequencing (NGS) technologies has revolutionized the cultivation-independent metagenomic approach, enabled researchers to obtain much more DNA information from highly complex microbial communities (Mardis 2008). These NGS platforms such as 454 pyrosequencing, Illumina, Solid<sup>TM</sup> systems, and Ion Torrent<sup>TM</sup> are much faster and cheaper than

the traditional Sanger method in DNA sequencing. In a recent study, Hong et al. (2012) used 454-pyrosequencing to analyze the airborne biotic contaminants in pig and poultry confinement buildings sampled from different climate conditions and found that the different livestock as well as production phase were associated with distinct airborne bacterial communities. In an another recent study, Boissy et al. (2014) used shotgun pyrosequencing metagenomic analyses of DNA from settled surface dusts from swine confinement facilities and grain elevators and found that the domain “Bacteria” predominates in bioaerosols followed by the domain “Eukaryota” and “Archaea”. The NGS based cultivation-independent metagenomic approach could next be applied to understand and determine how manure removal system and seasonal variations affect the bioaerosols in SCBs, which might ultimately be helpful in establishing better management strategies to minimize the potential health impacts on both livestock and humans working in this environment.

### **1.3. Airborne contaminants mitigation strategies**

The mitigation strategies of airborne contaminants from SCBs are classified mainly into two groups: prevention of pollutant formation and abatement of pollutants.

#### **1.3.1. Particulate matter mitigation**

The emissions of PM from SCBs can be reduced by preventing their formation (Martin *et al.* 1996). The use of feed additives, oil or water spraying, adequate ventilation and regular management of

manure all can minimize the PM emissions from SCBs (Maghirang *et al.* 1995). The use feed additives such as animal fat or vegetable oil has been shown to reduce both inhalable and respirable fractions of PM (Guarino *et al.* 2007). Peason and Sharples (1995) suggested that altering the shape of the feed, composition and its delivery system can reduce the PM generation from feed materials. The spraying of oil and water mixtures together with a feed enhanced by fat have been also shown to reduce the PM concentrations (Pedersen *et al.* 2000; Takai & Pedersen 2000). The use of filtration system has shown not only to remove PM efficiently (up to 99% efficiency), but also appears to be the cost effective option (Owen 1982a, b).

### **1.3.2. Gaseous compound mitigation**

The gaseous compounds in SCBs are mainly originated from feces and urine therefore the production of gaseous compounds can be reduced by modifying feed. It has been shown that for each percent reduction of crude protein content in feed can reduce NH<sub>3</sub> emissions by 9.5% (Le *et al.* 2009). The use of manure additive can also reduce the emission of gaseous compounds. Additions of nitrites and molybdates to manure have shown to reduce H<sub>2</sub>S emissions by inhibiting sulfate reducing bacteria and oxidizing sulfide (Predicala *et al.* 2008). Also, use of peroxides is shown to reduce the emissions of gaseous compounds derived from phenolics (Govere *et al.* 2005). Regular management of manure in pits under house floor has shown to reduce the emissions of NH<sub>3</sub> and other gaseous compounds (Guingand 2000). The use of essential oils has shown to reduce the intensity of odorous gaseous compounds (Kim *et al.* 2008a). Biofiltration system is also

used to reduce the emissions of gaseous compounds from SCBs, and it has been shown that biofilter allows a 64–69% decrease in  $\text{NH}_3$  and an 85–92.5% reduction in other odorous gaseous compounds (Sheridan *et al.* 2002). The removal efficiency of biofilter has been shown to fluctuate with seasonal variation, however still biofiltration is the most cost-effective technology for treating the ventilation exhaust air (Nicolai & Janni 1997).

### **1.3.3. Bioaerosol mitigation**

The approaches mentioned earlier for PM and gaseous compound mitigations such as the use of feed additives, oil or water spraying, adequate ventilation and the use of biofiltration system can also be used to reduce and control the emissions of bioaerosols from SCBs. Gore *et al.* (1986) reported a 27% reduction in concentrations of airborne bacteria, when 5% soybean oil was added to the feed. Kim *et al.* (2006) sprayed several biological additives in pig houses and found that soybean oil spray significantly reduced total dust, airborne bacteria and fungi until 24h in SCBs. Air scrubbers and biofilters have been also reported to reduce the emissions of bioaerosols from SCBs. Aarnink *et al.* (2011) reported a 70% reduction in concentrations of total bacteria by using a sulfuric acid scrubber. Although, biofilters have been shown very efficient and cost effective in reducing odorous gaseous compounds, these are not consistent in reducing microorganisms (Seedorf & Hartung 1999). This could be probably due to the emission of microorganisms to the ambient air which colonized on the surface of biofilter.

## 1.4. Objectives of this study

While much has been done to monitor the indoor airborne contaminants in SCBs (Hong *et al.* 2012; Nehmé *et al.* 2009; Nehme *et al.* 2008), relatively little is known about the factors influencing the abundance and community composition of bioaerosols in SCBs. Also, despite growing awareness of health risk in the neighboring resident community, the mitigation strategies of airborne contaminants emission from SCBs are poorly studied. Therefore, there is a need to thoroughly characterize the airborne contaminants present in SCBs, and to develop a viable mitigation strategy to reduce their emission levels from SCBs.

The present study provides a thorough research on characterization and mitigation of airborne contaminants present in SCBs. The aerial bioaerosols community structure and diversity were analyzed using NGS based cultivation-independent metagenomic approach, and a biofiltration system was used in this study to mitigate the emission of airborne contaminants from SCBs. This study essentially sets out to answer the following questions:

- 1) How does the abundance and composition of airborne biotic contaminants vary in bioaerosols of SCBs equipped with different types of manure removal system?
- 2) Are the abundance and composition of airborne biotic contaminants more similar in bioaerosol samples collected during similar season? If so, are differences in abundance and composition better explained by variation in microclimate variables?

3) How and to what extent the emissions of airborne biotic contaminants from SCBs influence by biofiltration system?

# **CHAPTER 2. Effect of Manure Removal System on Airborne Biotic Contaminants Present in Swine Confinement Buildings**

## **2.1. Manure removal system influences the abundance and composition of airborne biotic contaminants in swine confinement buildings**

### **2.1.1. Introduction**

In recent years, there has been an increase in the use of confined buildings for swine farming in all over the world. Because of the high animal densities in these confined environments waste excreted from the pigs and residual feed accumulate indoors leading to poor indoor air quality. As a result, animals and workers are exposed to large quantities of volatile odorous compounds and a variety of bioaerosols that may impact their health (Cole *et al.* 2000; Yao *et al.* 2011; Zejda *et al.* 1994).

Bacteria are one of the major constituent of bioaerosols within SCBs with a mean airborne concentration of around  $10^5$  cfu m<sup>-3</sup> (Chang *et al.* 2001; Nehme *et al.* 2008). Aerosol bacterial pathogens in SCBs can cause infectious and allergic diseases such as pneumonia, asthma, and rhinitis in workers and pigs (Donham *et al.* 1990; Olson & Bark 1996; Pearson & Sharples 1995; Whyte 1993). The known allergenic, toxic, and inflammatory responses are caused by exposure not only to the viable but also to the non-viable microorganisms present in the air (Robbins *et al.* 2000). Furthermore, the development and abundance of antibiotic resistance in microbes has been promoted due to antibiotics usage in swine farming (Blake *et al.* 2003; Zhu *et al.* 2013), which can become aerosolized within SCBs. Because the indoor air is regularly ventilated from SCBs to external environment, these airborne contaminants can pose a serious problem to public health. Tetracycline

is the most commonly used antibiotic in livestock production in Korea (KFDA 2006). Although the use tetracycline in animal feed is completely prohibited in Korea from July 2011, still the resistance of tetracycline is prevalent in aerosol samples of SCBs (Kumari & Choi 2014).

Previous studies estimating bacterial bioaerosol content and levels in SCBs have been carried out predominately by cultivation-dependent methods (Lee *et al.* 2006; Nehmé *et al.* 2009; Predicala *et al.* 2002; Yao *et al.* 2010). However, culture-based techniques contain an inherent bias, as only the viable microorganisms that can be grown in the selected culture are identified. There have been only few studies that have investigated the bacterial bioaerosol community in SCBs using culture-independent methods. Nehme *et al.* (2009; 2008) examined bacterial and archaeal biodiversity using culture-independent methods and found that bioaerosol populations bear close resemblance to the fecal microbiota of confined pigs. In another study, Kristiansen *et al.* (2012) also applied cultivation-independent molecular approaches to estimate the diversity and abundance of bioaerosolic bacteria and fungi in SCBs and suggested that swine feces are the primary source of the bacteria in the bioaerosols. In recent studies using next-generation sequencing methods, it has been further validated that a major portion of the bacterial bioaerosols in SCBs is originated from excreted feces and soiled bedding material (Hong *et al.* 2012; Kumari & Choi 2014). Despite these revelations that most of airborne bacteria in SCBs are originated from swine feces, still relatively little is known about the effect of manure removal systems on abundance and composition of airborne biotic contaminants in SCBs.

Hence, this study essentially sets out to answer the following questions using the more accurate 16S rRNA based pyrosequencing and quantitative PCR methods:

(1) How does the abundance of airborne biotic contaminants vary in bioaersols of SCBs equipped with the different types of manure removal system?

(2) What are the dominant bacterial taxa in bioaersols of SCBs and how does the bacterial community composition vary in SCB bioaersols equipped with the different types of manure removal systems?

## **2.1.2. Materials and Methods**

### **Characteristics of swine confinement buildings**

Samples were collected during the winter (January) season of 2013 from eight commercial swine farms in South Korea (Table 1). All sample collections took place in the growing/finishing rooms before the pigs were sent to the slaughterhouse. The ventilation methods employed in SCBs were mechanical ventilation (n=3) by exhaust fans on exit walls and natural ventilation (n=5) by means of a winch curtain. The number of animals housed in each sampling room ranged from 140 to 480, and the stocking density varied from 0.88 to 1.41 m<sup>2</sup>/ head (Table 1). The representative types of manure removal system equipped in these SCBs were deep-pit manure removal with slats (n=3), scraper removal system (n=3), and deep-litter bed system (n=2) (Figure 1). The deep pit manure system is composed of a deep manure pit under a fully or partially slatted floor. The manure is stored in the pit for a relatively long period of time before removal. The manure scraper removal system consists of a shallow manure pit with scrapers under a fully

slatted floor with the manure stored in shallow pit being removed several times a day from the SCBs. In the deep-litter bed system, pigs are kept on about 40-cm thick layer of a mixture of manure and litter such as sawdust, straw, or wood shavings. All pigs were fed with feeds from the Daehan Feed (Daehan Feed Co., Ltd., Korea) or from mills installed inside the farms.

### **Sample collection**

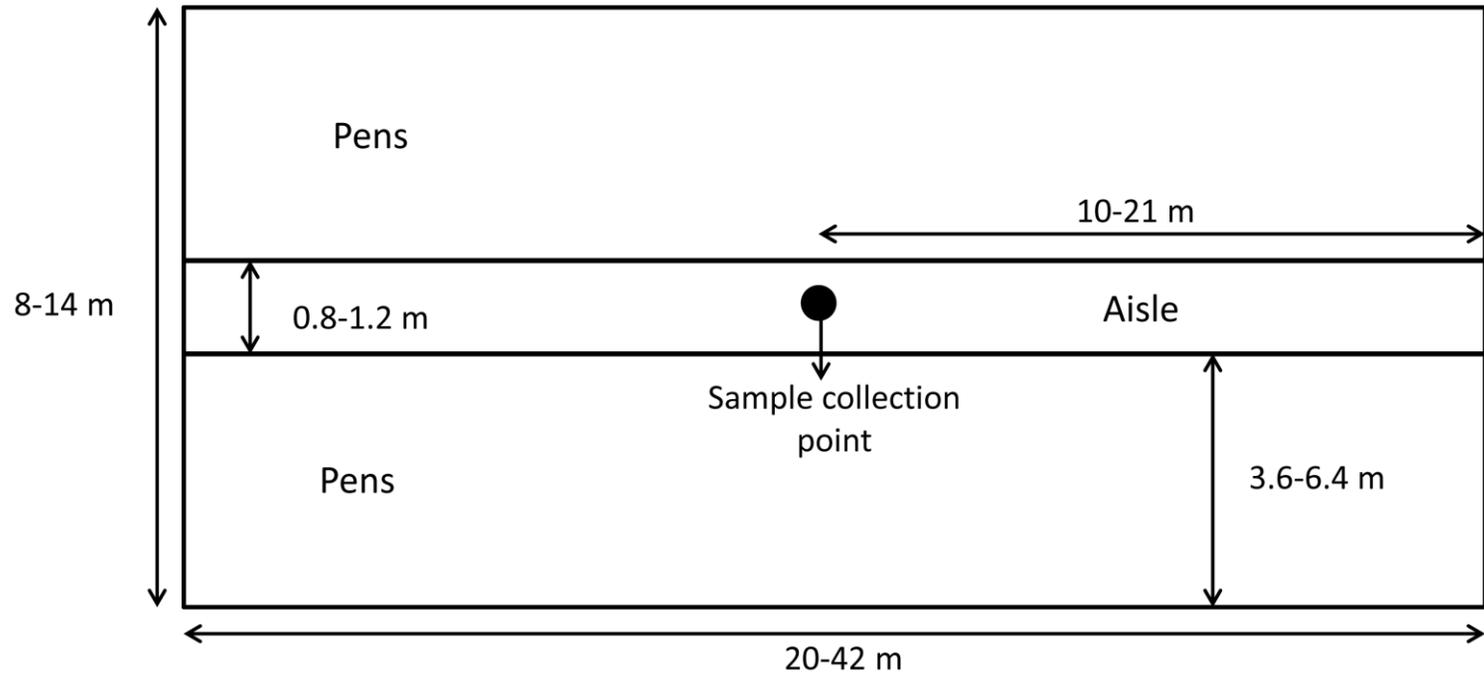
Aerosol samples were collected only once from the middle point in the aisle outside the pens at a height of 1.4 m above the floor (Figure 2). Air samples were captured on sterile 0.22- $\mu\text{m}$  cellulose nitrate filters (Fisher Scientific, Pittsburgh, PA) via a Gilian air sampler (Sensidyne Inc., Clearwater, FL, USA) (Figure A1) with a flow rate of 4  $\text{l min}^{-1}$  for 24 h. All components of the sampling system were sterilized in laboratory and aseptically assembled onsite prior sampling. All samples were immediately transported to the laboratory for subsequent molecular analyses. Blank filters were analyzed alongside sample filters to test for contamination, and following DNA extraction and amplification, blank filters were consistently found to be free of microbial contaminants.



**Figure 1.** Swine confinement buildings with (a) deep-pit manure removal with slats, (b) scraper removal system and (c) deep-litter bed system.

**Table 1.** Characteristics of the swine confinement buildings investigated in this study.

<b>Sample ID</b>	<b>Number of animals inside house</b>	<b>Stocking density m<sup>2</sup>/head</b>	<b>Manure removal system</b>	<b>Cleaning cycle</b>	<b>Ventilation mode</b>
F1	424	0.94	Deep-pit manure system with slats	About 6 months	Natural ventilation
F2	450	1.15	Deep-pit manure system with slats	About 6 months	Natural ventilation
F3	350	1.14	Deep-pit manure system with slats	About 6 months	Mechanical ventilation
F4	400	0.93	Manure removal by scraper	About 2 times in a day	Natural ventilation
F5	140	0.88	Manure removal by scraper	About 2 times in a day	Mechanical ventilation
F6	480	0.91	Manure removal by scraper	About 2 times in a day	Mechanical ventilation
F7	375	1.41	Deep-litter bed System	About 4 to 6 months	Natural ventilation
F8	352	1.13	Deep-litter bed system	About 4 to 6 months	Natural ventilation



**Figure 2.** Indoor plan view of aerosol collection point (black circle) in swine confinement buildings.

## **DNA extraction and quantitative PCR**

Bacterial DNA was extracted directly from the filters using the PowerSoil DNA isolation kit (MoBioLaboratories, Carlsbad, CA). Individual filters were aseptically cut into small pieces, loaded into the bead tube of the DNA extraction kit, and heated to 65 °C for 10 min followed by 2 min of vortexing. The remaining steps of the DNA extraction were performed according to the manufacturer's instructions. The purified DNA was resuspended in 50 µl of solution S6 (MoBio Laboratories) and stored at -20 °C until PCR amplification. The relative abundance of bacterial 16S rRNA genes and six tetracycline resistance genes (ribosomal protection proteins (RPP) class: tetO, tetQ, and tetW; Efflux class: tetB, tetH, and tetZ, refer to Levy et al. (1999) for the details on nomenclature) copy numbers were measured by quantitative PCR (qPCR) using the primers described in Table 2. The 16S rRNA genes and tetracycline resistance gene abundances were quantified against a standard curve generated from a plasmid containing the copies of respective genes, using a 10-fold serial dilution. The 20-µl qPCR mixtures contained 10 µl of 2× SYBR Green PCR Master Mix (Applied BioSystems, Foster City, CA, USA), 1.0 µl each of the 10 µM forward and reverse primers, and 7.0 µl of sterile, DNA-free water. Standard and environmental (ca. 1.0 ng) DNA samples were added at 1.0 µl per reaction. The reaction was carried out on an ABI Prism 7300 sequence detector (Applied Biosystems, Foster City, CA, USA) with an initial step of 95 °C for 10 min, followed by 40 cycles of denaturation (95 °C for 10 s), and primer annealing and extension (60 °C for 1 min). Dissociation curve analyses were performed to ensure the specificity of PCR, which included an increment of temperature

from 60 to 95 °C, at an interval of 0.5 °C for 5 s. Gene copy numbers were determined using a regression equation for each assay and relating the cycle threshold (CT) value to the known numbers of copies in the standards. The efficiencies of the qPCR were 90 to 95 % ( $R^2 > 0.991$ ). All qPCR reactions were run in quadruplicate with the DNA extracted from each sample.

### **PCR amplification and pyrosequencing**

The extracted DNA was amplified using primers targeting the V1 to V3 hypervariable regions of the bacterial 16S rRNA gene (Unno *et al.* 2010). The primers used for bacteria were V1-9F: 5'-X-ACGAGTTTGATCMTGGCTCAG-3' and V3-541R: 5'-X-AC-WTTACCGCGGCTGCTGG-3' (where X bar code is uniquely designed for each bioaerosol sample, followed by a common linker AC). Polymerase chain reactions were carried out under the following conditions: initial denaturation at 94 °C for 5 min, followed by 10 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C to 55 °C with a touchdown program for 45 s, and elongation at 72 °C for 90 s. This was followed by an additional 20 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 45 s, and elongation at 72 °C for 90 s. The amplified products were purified using the QIAquick PCR purification kit (Qiagen, CA, USA). Amplicon pyrosequencing was performed at Beijing Genome Institute (BGI), Hong Kong, China, using 454/Roche GS-FLX Titanium instrument (Roche, NJ, USA).

**Table 2.** Q-PCR primers used to quantify the abundance of 16S rRNA genes and tetracycline resistance genes.

Primer names	Target gene	Sequence (5'-3')	Resistance mechanism
338F	16S rRNA	ACT CCT ACG GGA GGC AGC	Not applicable
519R	gene	GWA TTA CCG CGG CKG	
TetB-FW	<i>tetB</i>	TAC GTG AAT TTA TTG CTT CGG	Efflux pumps (Aminov <i>et al.</i> 2002)
TetB-RV		ATA CAG CAT CCA AAG CGC AC	
TetH-FW	<i>tetH</i>	CAG TGA AAA TTC ACT GGC AAC	
TetH-RV		ATC CAA AGT GTG GTT GAG AAT	
TetZ-FW	<i>tetZ</i>	CCT TCT CGA CCA GGT CGG	
TetZ-RV		ACC CAC AGC GTG TCC GTC	
TetO-FW	<i>tetO</i>	ACG GAR AGT TTA TTG TAT ACC	Ribosomal protection proteins (Aminov <i>et al.</i> 2001)
TetO-RV		TGG CGT ATC TAT AAT GTT GAC	
TetQ-FW	<i>tetQ</i>	AGA ATC TGC TGT TTG CCA GTG	
TetQ-RV		CGG AGT GTC AAT GAT ATT GCA	
TetW-FW	<i>tetW</i>	GAG AGC CTG CTA TAT GCC AGC	
TetW-RV		GGG CGT ATC CAC AAT GTT AAC	

### **Analysis of pyrosequencing data**

The sequence data obtained by pyrosequencing were processed using mothur software (Schloss *et al.* 2009). Sequences shorter than 200 nucleotides with homopolymers longer than 8 nucleotides and all reads containing ambiguous base calls or incorrect primer sequences were removed. Next, the sequences were arranged against a SILVA alignment ([http://www.mothur.org/wiki/Alignment\\_database](http://www.mothur.org/wiki/Alignment_database)). Putative chimeric sequences were detected and removed via the Chimera Uchime algorithm contained within mothur (Edgar *et al.* 2011). All taxonomic classification was performed using mothur's version of the Ribosomal Database Project (RDP) Bayesian classifier, using a RDP training dataset ([http://www.mothur.org/wiki/RDP\\_reference\\_files](http://www.mothur.org/wiki/RDP_reference_files)) normalized to contain six taxonomic levels for each sequence at 80 % Naïve Bayesian bootstrap cutoff with 1000 iterations. All sequence data are available under the NCBI SRA accession no. SRP044637.

### **Statistical processing and analysis of results**

The variation in 16S rRNA gene and tetracycline gene abundances in SCBs equipped with different manure removal systems was assessed using a oneway ANOVA with Tukey's post-hoc tests for each pairwise comparison. All samples were standardized by random subsampling to 435 sequences per sample using the sub.sample command (<http://www.mothur.org/wiki/Sub.sample>) in mothur. Operational taxonomic units (OTUs) (at 97 % similarity) and rarefaction values were calculated using the mothur platform. OTU-based community similarity data was first square-root transformed to build the Bray–Curtis distance matrix. Nonmetric multidimensional

scaling (NMDS) was used to visualize the Bray–Curtis distances of bacterial community across all samples using primer PRIMER v6 (Clarke & Gorley 2006). To look at the effect of different types of manure collection system and ventilation type on bacterial community composition, an analysis of similarity (ANOSIM) with pairwise Bray–Curtis distance was performed with 999 random permutations and statistical significance was considered at  $P < 0.05$ . One-way ANOVA with Tukey’s post-hoc tests were conducted to determine whether the manure collection system had a significant impact on the relative abundance of bacterial taxa.

### **2.1.3. Results and discussion**

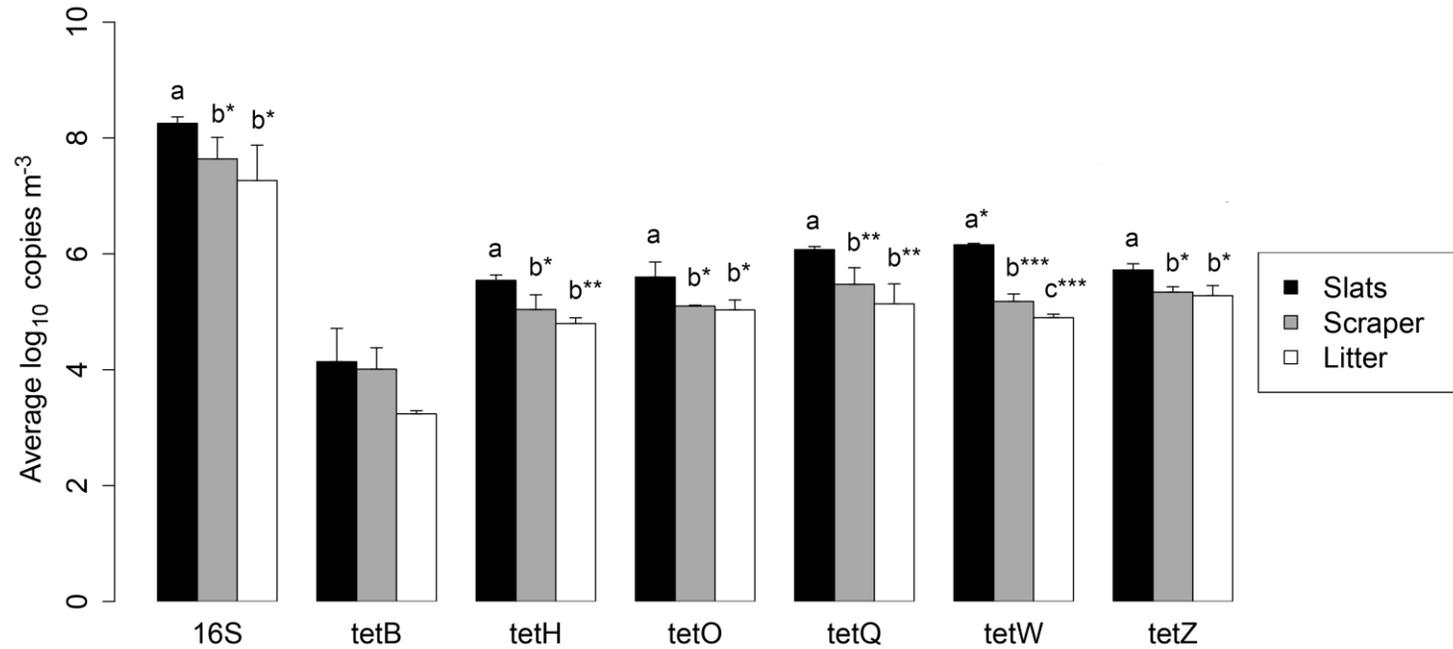
In this study, quantitative real-time PCR was used to evaluate the level of biotic contaminants and 454-pyrosequencing to characterize the bacterial bioaerosol community in SCBs. Pyrosequencing provided a comprehensive insight into the bacterial bioaerosols in SCBs.

#### **Effect of manure removal system on bacterial 16S rRNA and tetracycline gene abundances**

From the qPCR analyses, the bacterial 16S rRNA gene copy numbers differed in relation to manure removal system ( $P < 0.01$ ) (Figure 3), particularly between the deep-pit manure system with slats and manure removal system by scraper ( $P = 0.02$ ) and between deep-pit manure system with slats and deep-litter bed system ( $P = 0.01$ ). Airborne bacterial abundances peaked in bioaerosol samples collected from the SCBs equipped with deep-pit manure system with slats than

the scraper and litter systems (Figure 3). However, in a separate study involving different SCBs and culture-based methods, Kim *et al.* (2007) found that bacterial concentrations were highest in SCBs with a deep-litter bed system. The possible reasons for this discrepancy could be attributed to the difference in the techniques used to detect the bacterial concentrations and difference in the seasons of sample collection. Culture-independent methods are shown to be more accurate in determining the total airborne bacterial concentrations and estimate up to 1000 times higher than the concentration of airborne culturable bacteria (Nehme *et al.* 2008).

The abundance of tetracycline resistance genes were significantly higher in SCBs equipped with a deep-pit manure removal system with slats in comparison to scraper and deep-litter bed system, except for tetB gene (Figure 3). Swine manure is known to be a 'hot spot' of bacteria carrying tetracycline resistance genes (Heuer *et al.* 2002; Smalla *et al.* 2000), and the increased concentration of both 16S rRNA and tetracycline genes in bioaerosol samples of SCBs with the deep-pit system could be the product of longer storage of manure in the pits before removal.



**Figure 3.** Abundance of 16S rRNA and tetracycline resistance genes in swine confinement buildings equipped with different manure removal systems. Tukey pairwise comparisons are shown; different letters denote significant differences between groups. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$

## **Effect of manure removal system on bacterial community composition and diversity**

From 454-pyrosequencing, a total of 14,315 good quality bacterial sequences (with an average length of 309 bp) were obtained from the 8 samples, with an average of 1789 sequences per sample and with coverage ranging from 435 to 3013 reads per sample (Table 3). Of the 14,315 good-quality sequences, a total of 976 OTUs were obtained at  $\geq 97$  % sequence similarity level. The average number of OTUs per sample was  $204 \pm 98$  (standard deviation [SD]), ranging from 84 to 344 OTUs (Figure 4).

The manure removal system was found to strongly influence the community composition and relative abundances of the major bacterial phyla in SCBs. The most abundant bacterial phylum was *Firmicutes* with 72.4 % of the sequences, followed by *Actinobacteria* (10.7 %), *Bacteroidetes* (9.8 %), and *Proteobacteria* (5.2 %) (Figure 5). Of these most abundant phyla, the relative abundance of *Firmicutes*, *Actinobacteria*, and *Proteobacteria* were found to be significantly different in SCBs with different manure removal systems (Figure 5). The relative abundance of *Firmicutes* was significantly higher ( $P=0.01$ ) in SCBs with slats and scraper than in deep-litter bed (Figure 5). *Actinobacteria* and *Proteobacteria* were found to be significantly abundant ( $P < 0.05$ ) in SCBs with deep-litter bed system compared to in the slats or scraper systems (Figure 5). The relative abundance of bacterial genera within the pre dominant phyla *Firmicutes* and *Actinobacteria* were further evaluated, and the bacterial bioaerosol community of SCBs was found to be predominantly made up by genera *Lactobacillus* (13.1 %), *Clostridium* (9.7 %), *Corynebacterium* (7.7 %),

*Staphylococcus* (3.3 %), and *Streptococcus* (2.7 %). Out of these, *Corynebacterium* was particularly enriched in SCBs with a deep-litter bed system ( $P < 0.01$ ) exhibiting an approximately 30-fold increase over SCBs with slats and scraper system.

The phylum *Firmicutes* has been shown to dominate SCB environments (Hong *et al.* 2012; Kumari & Choi 2014; Lee *et al.* 2006; Olson & Bark 1996). The predominant *Firmicutes* genera *Lactobacillus*, *Clostridium*, *Staphylococcus*, and *Streptococcus* are found to be commonly associated with the gastrointestinal tract of pig (Leser *et al.* 2002), suggesting that swine feces is the primary source of the phylum *Firmicutes* in the SCB bioaerosol. The higher relative abundance of the phylum *Firmicutes* in SCBs with the deep-pit system could be the result of longer storage of manure in the pits before removal. On the other hand, the dominance of *Actinobacteria* in the deep-litter bed system could be explained by the facts that they are capable to multiply in mixture of feces and litter in favorable temperature and aeration (Fries *et al.* 2005; Martin *et al.* 1998).

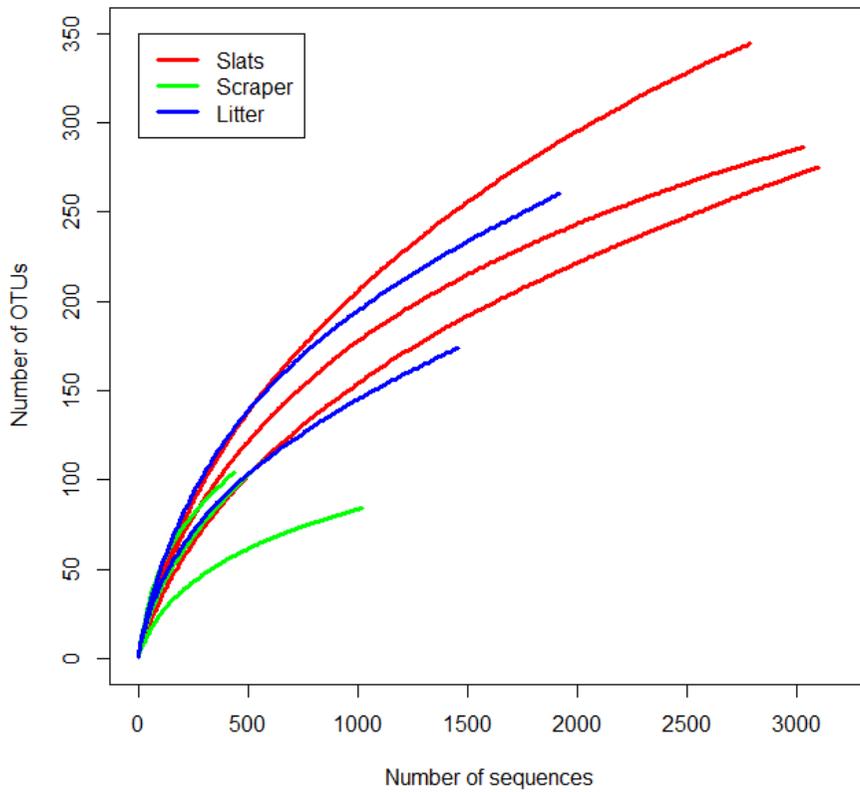
Bacterial diversity levels in bioaerosols were high in SCBs with deep-litter bed and deep pit with slats in comparison to scraper (Table 3). However, bacterial species-level richness (i.e., number of OTUs) did not differ among different manure removal systems ( $P = 0.27$ ). Similarly, none of the diversity indices differed between SCBs with different manure removal systems (Shannon  $P = 0.69$ , inverse Simpson  $P = 0.90$ , Chao  $P = 0.25$ , and Ace  $P = 0.36$ ). An NMDS plot of Bray–Curtis distance showed significant (ANOSIM:  $R = 0.68$ ,  $P < 0.01$ ) discrimination in composition of the airborne bacterial communities between samples collected from SCBs equipped with different manure

removal systems (Figure 6a), whereas there was no significant effect of air ventilation type on bacterial community composition (ANOSIM:  $R = -0.005$ ,  $P = 0.47$ ). The network analysis in Figure 6b highlights that bacterial communities in SCBs with the same manure removal system were more similar to each other than to communities of a different system with some overlap between slats and scraper systems. The network-based analysis complements the Bray–Curtis distance-based community analysis in Figure 6a, as the network was used to map the airborne bacterial community composition to specific manure removal systems. These results are in agreement with previous findings that a significant portion of the bioaerosols in confinement buildings originated from excreted feces and soiled bedding material (Duan *et al.* 2009; Dumas *et al.* 2011; Kumari & Choi 2014; Nehme *et al.* 2008; Nonnenmann *et al.* 2010). However, samples collected from SCBs with the scraper system did not cluster closely; one possible explanation for this could be that in the scraper system, manure can be removed from the swine house completely several times a day which results in a variation in bacterial community composition within the samples collected from the SCBs with the scraper system.

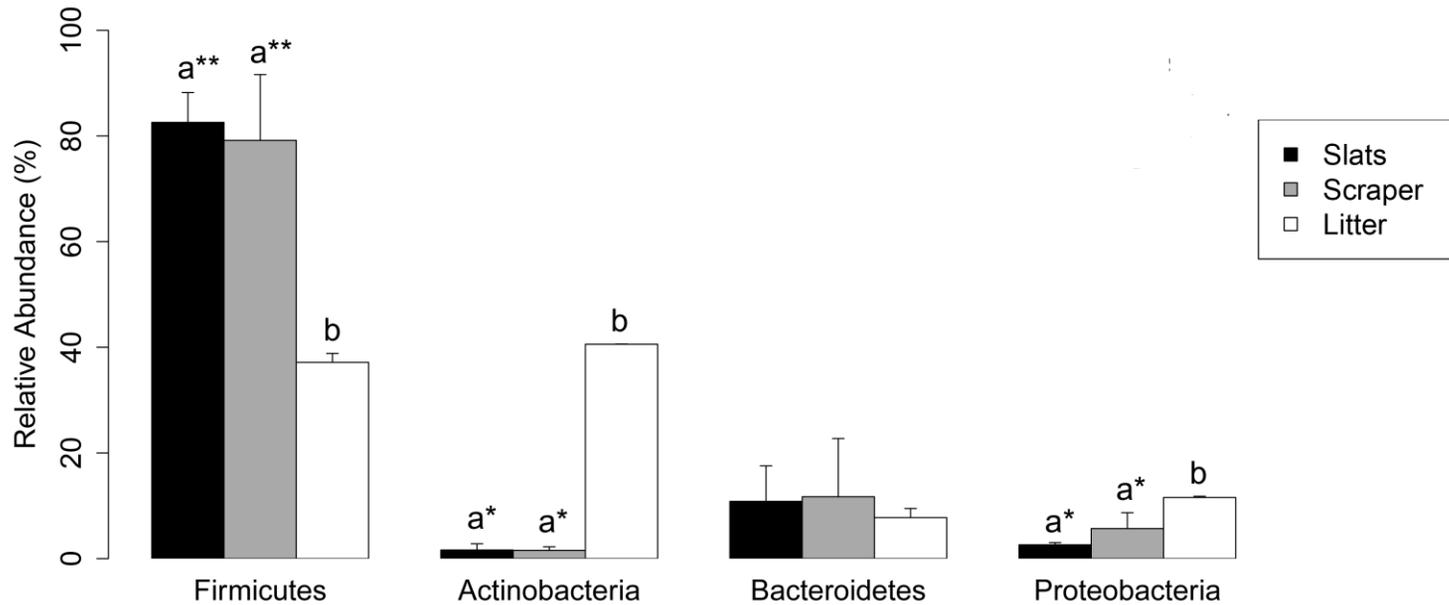
**Table 3.** Diversity indices of bioaerosol samples collected from swine confinement buildings.

ID	Manure removal system	Number of sequences	Normalized to 435 reads						
			OTU richness	PD <sup>a</sup>	Coverage	Shannon	Simpson	Ace	Chao
F1	Deep-pit manure system with slats	3028	119	7.624	0.8229	3.60	0.078	548.7	265.3
F2	Deep-pit manure system with slats	2787	118	7.682	0.8344	3.45	0.103	338.5	234.1
F3	Deep-pit manure system with slats	3103	100	6.814	0.8574	2.92	0.173	191.5	205.0
F4	Manure removal by scraper	573	96	6.490	0.8758	3.19	0.139	274.4	206.0
F5	Manure removal by scraper	1014	50	4.263	0.9448	2.05	0.310	103.4	80.6
F6	Manure removal by scraper	435	105	7.232	0.8919	4.03	0.029	190.8	188.1
F7	Deep-litter bed system	1917	128	8.601	0.8390	3.79	0.073	318.6	211.2
F8	Deep-litter bed system	1458	100	7.271	0.8850	3.46	0.084	170.9	158.3

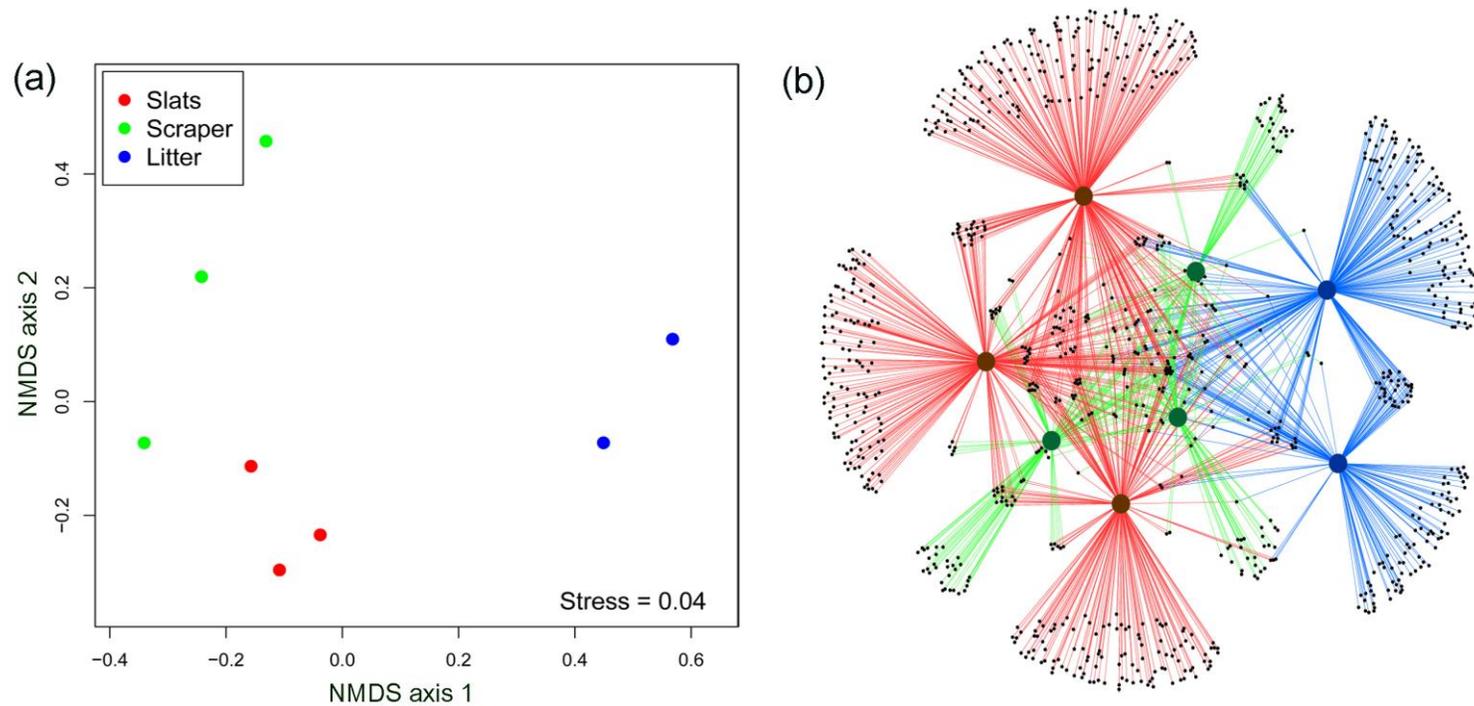
<sup>a</sup>PD: Phylogenetic diversity.



**Figure 4.** Rarefaction curves comparing airborne bacterial communities in swine confinement buildings with different manure removal systems.



**Figure 5.** Relative abundance (mean±SD) of dominant airborne bacterial phyla in swine confinement buildings equipped with different manure collection systems. Tukey pairwise comparisons are shown; different letters denote significant differences between groups. \* $P < 0.05$ ; \*\* $P = 0.01$



**Figure 6.** (a) NMDS plot of the pairwise Bray–Curtis distance matrix displaying OTU clustering of airborne bacterial communities in swine confinement buildings by manure removal systems. (b) Network analysis of the airborne bacterial communities in SCBs according to manure removal system.

#### **2.1.4. Conclusions**

In conclusion, the manure removal system strongly influenced the abundance and community composition of airborne biotic contaminants of SCBs. The airborne biotic contaminants were most abundant in SCBs equipped with a deep pit with slats. *Firmicutes* and *Actinomycetes* appear to be the dominant bacterial phyla in SCBs, and a comparison of relative abundances of these phyla together with dominant genera strongly supports the concept that individual bacterial lineages found in SCBs are enriched to specific manure removal systems. The present study represents a first glimpse of the airborne biotic contaminants of SCBs with different types of manure removal systems using next-generation sequencing methods. Based on the results of this study, better management practices and regulations can be designed to minimize the potential health impact on both livestock and humans working in this environment.

**CHAPTER 3. Seasonal  
Variability in Airborne Biotic  
Contaminants in Swine  
Confinement Buildings**

## **3.1. Seasonal variability in bacterial bioaerosols and antibiotic resistant genes in swine confinement buildings**

### **3.1.1. Introduction**

The intensification of pig farming in confined buildings with high animal densities can lead to poor indoor air quality. Microbial decomposition of proteinaceous waste products in feces and urine results in elevated concentrations of volatile organic compounds, NH<sub>3</sub>, and sulfides (O'Neill & Phillips 1992), whereas feed materials, skin debris, bedding material, and dried manure generate airborne particulates that carry adsorbed microorganisms and endotoxins (Cambra-López *et al.* 2010). Poor indoor air quality in SCBs affects both animal and human health. For example, airborne particulates can deposit in nasal channels and the respiratory tract and cause damage to lung tissues (Carpenter 1986). Furthermore, some airborne bacteria and gases such as NH<sub>3</sub>, H<sub>2</sub>S (from the manure), and CO<sub>2</sub> (pig activity) can cause or trigger chronic respiratory tract inflammation in workers and pigs (Charavaryamath & Singh 2006; Choudat *et al.* 1994; Cormier *et al.* 1990; Donham *et al.* 1989; Dosman *et al.* 2004; Heederik *et al.* 1991; Israel-Assayag & Cormier 2002; Mackiewicz 1998).

Microclimatic variables can influence the formation of aerosols containing microorganisms. There are great variations in the outside temperature in South Korea (-7 to 1°C in winter and from 22°C to 30°C in summer) (Korea 2008), so maintaining an optimal indoor temperature in SCBs can be challenging. Typically in the winter, all of the openings are closed, and the ventilation rate has to be minimal to

reduce the heat loss. This low ventilation rate could induce an increased concentration of airborne contaminants. In contrast, the ventilation is maximal during the summer, thus diminishing the indoor temperature and contributing to driving the indoor air outside the SCBs.

Bacteria constitute a huge proportion of organisms within bioaerosols in SCBs, with a mean concentration of  $10^5$  cfu m<sup>-3</sup> (Chang *et al.* 2001; Nehme *et al.* 2008). While much has been done to monitor the indoor airborne biotic contaminants in SCBs (Hong *et al.* 2012; Nehmé *et al.* 2009; Nehme *et al.* 2008), relatively little is known about the seasonal dynamics of airborne biotic contaminants and their interaction with microclimate parameters in SCBs. Nehme *et al.* (2009; 2008) examined the seasonal variability of airborne bacterial and archaeal communities in SCBs and found that, although the microbial abundances were significantly higher during the winter, the biodiversity was similar in each SCB during both the winter and summer seasons. However, these studies used low-resolution molecular fingerprinting tools, which lacked the coverage and depth of high-throughput sequencing methods. In a recent study, Hong *et al.* (2012) used 454-pyrosequencing to analyze the airborne biotic contaminants in pig and poultry confinement buildings sampled from different climate conditions and found that the different livestock as well as production phase were associated with distinct airborne bacterial communities; however, they did not evaluate the effect of microclimate variables on bacterial bioaerosol communities.

The usage of antibiotics in swine farms has promoted the development and abundance of antibiotic resistance in microbes (Blake *et al.* 2003; Zhu *et al.* 2013), which can become aerosolized within the

SCBs. Antibiotic resistant genes (ARGs) can be transferred to pathogens through transformation or phage-mediated transduction, and/or by conjugation, posing a serious threat to public health. Horizontal gene transfer plays important roles in the evolution and transmission of ARGs between bacterial species and includes the movement of ARGs from fecal bacteria to environmental bacteria, as well as the reverse; that is, emergence of novel mechanisms of acquired resistance in pathogens, ARGs that originally were present in harmless bacteria (Baquero *et al.* 2008). Tetracycline was chosen for this study because it is the most widely used broad spectrum antibiotic in livestock production worldwide, and is particularly prevalent in pig production (Delsol *et al.* 2003). The mechanism by which bacteria resist tetracycline antibiotics is heavily biased by ecological niche (Gibson *et al.* 2014), and compared to the existing literature on tetracycline resistance genes ( $Tc^R$ ) in soils and in water, relatively little is known regarding  $Tc^R$  genes in aerosols of SCBs (Hong *et al.* 2012; Ling *et al.* 2013). Three  $Tc^R$  genes (*tetB*, *tetH*, *tetZ*) encoding efflux proteins (EFP), and three others (*tetO*, *tetQ*, *tetW*) encoding ribosomal protection proteins (RPP) were selected for this study because these genes have been detected in aerosol of SCBs (Hong *et al.* 2012) and because these  $Tc^R$  genes encode two main mechanisms of bacterial resistance to tetracycline, which have been found associated with bacteria of public health interest (Chopra & Roberts 2001; Roberts *et al.* 2012; Santamaría *et al.* 2011).

Therefore, the aim of this study was to answer the following questions using the Illumina Hiseq sequencing of the V3 region of the 16S rRNA gene and qPCR of both 16S rRNA and  $Tc^R$  genes:

(1) How does the bacterial bioaerosol community composition and diversity vary in SCBs during the winter and summer seasons?

(2) Does the abundance of 16S rRNA and Tc<sup>R</sup> genes vary in SCBs during the winter and summer seasons?

(3) What are the major microclimate variables affecting the abundance, community composition, and diversity of airborne biotic contaminants in SCBs?

### **3.1.2. Materials and methods**

#### **Characteristics of animal confinement buildings**

The study was conducted in the winter (January) and summer (June) of 2013 on seven commercial pig farms located in six South Korean provinces. All the commercial pig farms sampled in this study are privately owned. Permission to access privately owned farms was given by the farm owners and for future permissions we can contact the owners. All samples were collected in the growing/finishing houses of SCBs. The average outdoor temperature across all of South Korea ranges from  $-7$  to  $1^{\circ}\text{C}$  in winter and  $22^{\circ}\text{C}$  to  $30^{\circ}\text{C}$  in summer. Temperature differences among the six provinces were less than  $2^{\circ}\text{C}$  (Korea 2008). Ventilation in SCBs was mechanical by exhaust fans on walls. The number of animals kept in each sampling room ranged from 140 to 480, and the stocking density varied from 0.88 to 1.41  $\text{m}^2/\text{head}$ . The age of the pigs varied from 67-150 days in each sampling room at the time of sampling. The manure removal system was deep-pit with slats, and the cleaning cycle varied from 4-6 months.

#### **Microclimate variables**

The microclimate variables were measured from three points in the aisle outside the pens at every 8 h interval till 24 h, and all the microclimate variables were reported as averages corresponding to each sampling period. Air temperature and relative humidity were measured with a hygrothermograph (SK-110TRH, SATO, Tokyo, Japan). Air speed was measured with an anemometer (model 6112, KANOMAX, Osaka, Japan). Particulate matter concentrations ( $\mu\text{g m}^{-3}$ ) were measured using an aerosol mass monitor (GT-331, SIBATA, Soka-city, Japan). The mass concentrations of PM10 (PM average aerodynamic diameter  $\leq 10 \mu\text{m}$ ), PM2.5 (PM mean aerodynamic diameter  $\leq 2.5 \mu\text{m}$ ), PM1 (PM mean aerodynamic diameter  $\leq 1 \mu\text{m}$ ), and total suspended particles (TSP) were obtained simultaneously, at a flow rate of  $2.83 \text{ L min}^{-1}$ . The concentrations of  $\text{NH}_3$ ,  $\text{H}_2\text{S}$  and  $\text{CO}_2$  were measured by gas detector tubes (Gastec Co., Ltd., Kanagawa, Japan).

### **Sample collection and DNA extraction**

Aerosol samples were collected from the middle point in the aisle outside the pens at a height of 1.4 m above the floor (Figure 2). Air samples were captured on sterile  $0.22\text{-}\mu\text{m}$  cellulose nitrate filters (Fisher Scientific, Pittsburgh, PA) via vacuum filtration with a flow rate of  $4 \text{ l min}^{-1}$  for 24 h. The cellulose nitrate filters were kept at  $4^\circ\text{C}$  until processing in the laboratory. Bacterial DNA was extracted directly from the filters using the PowerSoil DNA isolation kit (MoBio Laboratories, Carlsbad, CA). Individual filters were aseptically cut into small pieces, loaded into the bead tube of the DNA extraction kit, and heated to  $65^\circ\text{C}$  for 10 min followed by 2 min of vortexing. The remaining steps of the DNA extraction were performed according to

the manufacturer's instructions. The purified DNA was resuspended in 50 µl of solution S6 (MoBio Laboratories) and stored at -20°C until PCR amplification. Blank filters were analyzed alongside sample filters to test for contamination, and following DNA extraction and amplification, blank filters were consistently found to be free of microbial contaminants.

### **Illumina sequencing and data processing**

The extracted DNA was amplified using primers 338F (5'-XXXXXXXXX-GTACTCCTACGGGAGGCAGCAG-3') and 533R (5'-TTACCGCGGCTGCTGGCAC-3') targeting the V3 region of bacterial 16S rRNA ('X' denotes 8-mer barcode sequence) (Huse *et al.* 2008). Paired-end sequencing was performed at Beijing Genome Institute (BGI) (Hongkong, China) using 2 x 150 bp Hiseq2500 (Illumina) according to the manufacturer's instructions. Library preparation, sequencing, and initial quality filtering were performed as described previously (Zhou *et al.* 2011). The sequence data obtained by Illumina Hiseq2500 sequencing were processed using mothur (Schloss *et al.* 2009). Paired-end sequences were assembled, trimmed, and filtered in mothur. Next, the sequences were aligned against a SILVA alignment (<http://www.arb-silva.de/>). Putative chimeric sequences were detected and removed via the Chimera Uchime algorithm contained within mothur (Edgar *et al.* 2011). Sequences were denoised using the 'pre.cluster' command in mothur, which applies a pseudo-single linkage algorithm with the goal of removing sequences that are likely due to sequencing errors (Huse *et al.* 2010). All sequences were classified using the EzTaxon-e database ([43](http://eztaxon-</a></p></div><div data-bbox=)

e.ezbiocloud.net/) (Kim *et al.* 2012), using the classify command in mothur at 80% Naïve Bayesian bootstrap cutoff with 1000 iterations. Sequence data were deposited in SRA at NCBI with the accession number of SRP039383.

### **Quantification of 16S rRNA and Tc<sup>R</sup> genes**

A real-time polymerase chain reaction was used to quantify bacterial 16S rRNA and six Tc<sup>R</sup> genes (RPP class: *tetO*, *tetQ* and *tetW*; EFP class: *tetB*, *tetH* and *tetZ*, refer Levy et al. (1999) for the details on nomenclature) using the SYBR Green approach with the primers described in Table S1. The copy numbers of 16S rRNA and Tc<sup>R</sup> genes in bioaerosol samples were measured against the standard curves of plasmids containing copies of the respective genes, using a 10-fold serial dilution. All reactions were conducted in triplicate with the 20 µl qPCR mixtures containing 10 µl of 2X SYBR Green PCR Master Mix (Applied BioSystems, Foster City, CA, USA), 1.0 µl each of the 10 µM forward and reverse primers, and 7.0 µl of sterile, DNA-free water. Standard and bioaerosol (ca. 1.0 ng) DNA samples were added at 1.0 µl per reaction. The reaction was carried out on an ABI Prism 7300 sequence detector (Applied Biosystems, Foster City, CA, USA) with an initial step of 95°C for 10 min, followed by 40 cycles of denaturation (95°C for 10 s), and primer annealing and extension (60°C for 1 min). Dissociation curve analysis was performed to ensure the specificity of PCR, which included an increment of temperature from 60°C to 95°C at an interval of 0.5°C for 5 s. Gene copy numbers were determined using a regression equation for each assay and relating the cycle threshold (CT) value to the known numbers

of copies in the standards. The correlation coefficients of standard curves ranged from 0.957 to 0.996.

### **Statistical processing and analysis of results**

Rarefaction curves and diversity indices were generated using mothur, with the bacterial phylotype (OTU) defined here at 97% threshold of 16S rRNA gene sequence similarity. Phylogenetic diversity (PD) was calculated as Faith's PD (Faith 1992), the total phylogenetic branch length separating OTUs in each rarefied sample. To allow for robust comparisons among samples containing different numbers of sequences, phylotype richness and phylogenetic diversity were calculated based on samples rarefied to contain 15,909 sequences. To test for differences in relation to season on OTU richness, PD, 16S rRNA and Tc<sup>R</sup> genes abundances, we used a t-test for normal data and the Wilcoxon rank-sum test for non-normal data. We used the same procedure to test whether the relative abundance of the most abundant phyla differed across seasons.

To avoid including collinear variables in further analyses, we used a Spearman's correlation matrix and highly correlated (Spearman's  $r \geq 0.8$ ) microclimatic variables were removed from further analysis. Non-metric multidimensional scaling (NMDS) was generated based on pairwise Bray-Curtis dissimilarities between samples using the vegan R package (Oksanen *et al.* 2007). The analysis of similarity (ANOSIM) function in the vegan R package with 999 permutations was used to test for differences in bacterial communities among the winter and summer season. The vectors of microclimate variables were fitted onto ordination space (Bray–Curtis NMDS) to

detect possible associations between patterns of community structure and microclimate variables using the ‘envfit’ function of the vegan R package, and statistical significance was evaluated among 999 random permutations. Analyses for Venn diagram generation were performed using the mothur, and the Venn diagram was plotted using R package VennDiagram (Chen & Boutros 2011). Differentially abundant bacterial genera between the winter and summer seasons were identified using a parametric approach (Metastats) (White *et al.* 2009). All statistical analysis, graphs, and ordinations were produced using R version 3.0.2 (RDevelopmentCoreTeam 2008).

### **3.1.3. Results and discussion**

In this study, Illumina sequencing was used to provide a comprehensive insight into the bacterial bioaerosol community composition and diversity in SCBs. The use of high-throughput molecular sequencing methods revealed indoor microbial biodiversity that was previously difficult or impossible to observe (Sogin *et al.* 2006).

#### **Season variations in microclimate variables and bacterial bioaerosols diversity**

The means of microclimate variables in the SCBs during the winter and summer seasons are presented in Table 4. All of the microclimate variables in the SCBs differed significantly between the winter and summer season samples ( $P < 0.05$ ; Table 4), except total suspended particles,  $\text{NH}_3$  and  $\text{H}_2\text{S}$  ( $P > 0.05$ , Table 4). Spearman's correlation matrix showed highest correlation between PM10 and TSP

( $r = 0.93$ ; Table 5). Temperature and airspeed were the next most correlated variables ( $r = 0.9$ ; Table 5) followed by  $\text{NH}_3$  and  $\text{CO}_2$  ( $r = 0.82$ ; Table 2) and  $\text{PM}_{2.5}$  and  $\text{PM}_1$  ( $r = 0.81$ ; Table 5). Therefore, we removed  $\text{PM}_{10}$ , temperature,  $\text{PM}_1$  and  $\text{NH}_3$ , and used the remaining six microclimate variables, (i.e. airspeed, relative humidity,  $\text{PM}_{2.5}$ , TSP,  $\text{H}_2\text{S}$  and  $\text{CO}_2$ ) for further analyses.

From the 14 samples, we obtained 13,597 OTUs at 97% similarity from 497,607 good-quality sequences. The average number of OTUs per sample was  $2442 \pm 910$  (standard deviation [SD]), ranging from 1287 to 4045 OTUs. To compare diversity levels and community profiles between samples controlling for differences in sequencing depth, samples were compared at the same sequencing depth (15,909 randomly selected sequences per sample). At this depth of coverage, bacterial richness ( $P < 0.01$ ; Figure 7a) and phylogenetic diversity levels ( $P = 0.01$ ; Figure 7b) were significantly higher in winter in comparison to summer. This result is in contrast with the findings of Nehme et al.(2008), who examined the influence of seasonal variation on bacterial biodiversity in SCBs and found that biodiversity was unchanged between different seasons of the year. One of the possible explanations for this discrepancy in results could be that Nehme et al. (2008) analyzed very limited number of sequences for estimating the bacterial bioaerosol diversity. Furthermore, Nehme et al. (2008) used denaturing gradient gel electrophoresis and 16S-cloning-and-sequencing approach to characterize the bacterial bioaerosol community, which lack resolution and throughput, respectively, compared to next-generation sequencing based methods (Bartram *et al.* 2011; Bent *et al.* 2007; MacLean *et al.* 2009). Spearman's correlation

coefficients showed a significant negative correlation between air speed and both OTU richness and PD of bacterial bioaerosol communities (Table 6). Whereas, PM<sub>2.5</sub> and TSP were positively correlated to OTU richness and PD (Table 6). Airspeed has been shown to impact the diversity of indoor bacterial communities (Kembel *et al.* 2014), and these results suggest that the higher diversity levels in the winter season are likely to be a function of ventilation rate and particulate matter, as during summer under high ventilation rates, more airborne particulate matter carrying bacteria would be transferred out of the SCBs.

**Table 4.** Seasonal means ( $\pm$ SD) of microclimate variables in swine confinement buildings.

<b>Variable</b>	<b>Temp., °C</b>	<b>Relative humidity , %</b>	<b>Air speed, m/s</b>	<b>PM10, <math>\mu\text{g m}^{-3}</math></b>	<b>PM2.5, <math>\mu\text{g m}^{-3}</math></b>	<b>PM1, <math>\mu\text{g m}^{-3}</math></b>	<b>TSP, <math>\mu\text{g m}^{-3}</math></b>	<b>NH<sub>3</sub>, mg/L</b>	<b>H<sub>2</sub>S, mg/L</b>	<b>CO<sub>2</sub>, mg/L</b>
<b>Season</b>										
Winter	20.8 $\pm$ 5.5	68.5 $\pm$ 18.6	0.02 $\pm$ 0.02	470.8 $\pm$ 468.8	48.4 $\pm$ 27	25.7 $\pm$ 20.1	1252.2 $\pm$ 1320.5	24.4 $\pm$ 22.7	0.36 $\pm$ 0.34	2833.1 $\pm$ 1433.6
Summer	31.5 $\pm$ 4.2	86.3 $\pm$ 13.7	0.18 $\pm$ 0.06	77.5 $\pm$ 19.5	17.1 $\pm$ 8.2	7.6 $\pm$ 4.2	130.8 $\pm$ 34.2	14.9 $\pm$ 12.1	0.41 $\pm$ 0.43	1242.6 $\pm$ 423.7
<i>P</i> -value <sup>a</sup>	< 0.001	0.003	< 0.001	0.04	0.03	0.03	0.07	0.21	0.82	0.03

<sup>a</sup>For each variable, *P*-value was used to determine the significance of means among the two seasons.

**Table 5.** Spearman rank correlations between measured microclimatic variables in SCBs.

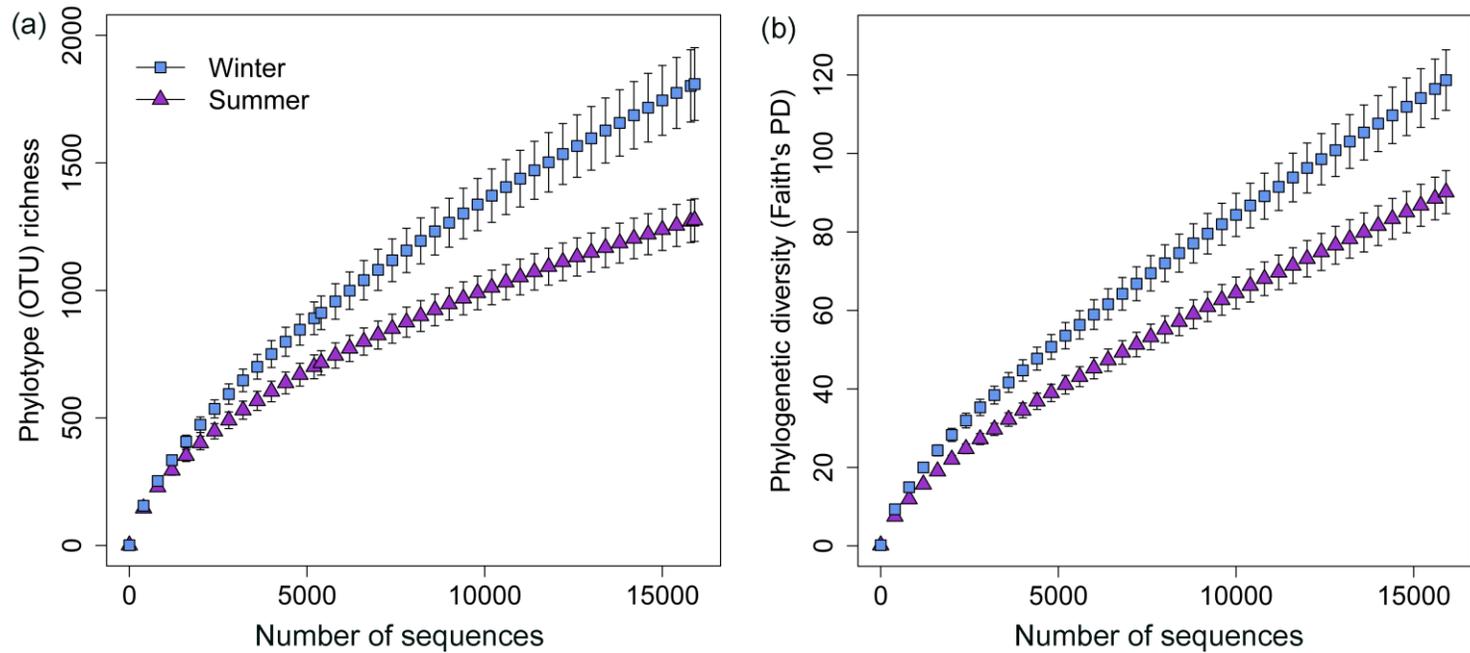
<b>Microclimate variables</b>	<b>Temp.</b>	<b>Relative humidity</b>	<b>Air speed</b>	<b>PM10</b>	<b>PM2.5</b>	<b>PM1</b>	<b>TSP</b>	<b>NH<sub>3</sub></b>	<b>H<sub>2</sub>S</b>	<b>CO<sub>2</sub></b>
Temp.	1									
Relative humidity	0.26	1								
Air speed	0.9***	0.3	1							
PM10	-0.52	-0.46	-0.39	1						
PM2.5	-0.69**	-0.35	-0.7**	0.72**	1					
PM1	-0.63*	-0.17	-0.64*	0.34	0.81***	1				
TSP	-0.39	-0.48	-0.23	0.93***	0.53	0.18	1			
NH <sub>3</sub>	-0.06	0.02	-0.28	-0.13	0.12	-0.02	-0.15	1		
H <sub>2</sub> S	0.21	-0.11	-0.06	-0.11	0.04	-0.11	-0.15	0.68**	1	
CO <sub>2</sub>	-0.26	-0.02	-0.46	0.19	0.48	0.3	0.14	0.82***	0.44	1

\* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

**Table 6.** Spearman rank correlations between microclimatic variables, diversity and abundances of airborne biotic contaminants in SCBs.

Microclimate variables	OTU richness	PD	16S rRNA gene	<i>tetB</i>	<i>tetH</i>	<i>tetZ</i>	<i>tetO</i>	<i>tetQ</i>	<i>tetW</i>
Relative humidity	-0.56*	-0.52	-0.4	-0.24	-0.26	-0.24	-0.47	-0.3	-0.39
Air speed	-0.7**	-0.71**	-0.78***	-0.14	-0.69**	-0.52	-0.61*	-0.68**	-0.73**
PM2.5	0.57*	0.57*	0.78***	0.24	0.62*	0.66*	0.58*	0.79***	0.79***
TSP	0.08	0.06	0.55*	0.45	0.56*	0.57*	0.64*	0.58*	0.6*
H <sub>2</sub> S	0.35	0.31	0.09	-0.11	-0.25	0.1	-0.19	-0.03	-0.07
CO <sub>2</sub>	0.42	0.4	0.56*	-0.24	0.32	0.63*	0.41	0.6*	0.49

\* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .



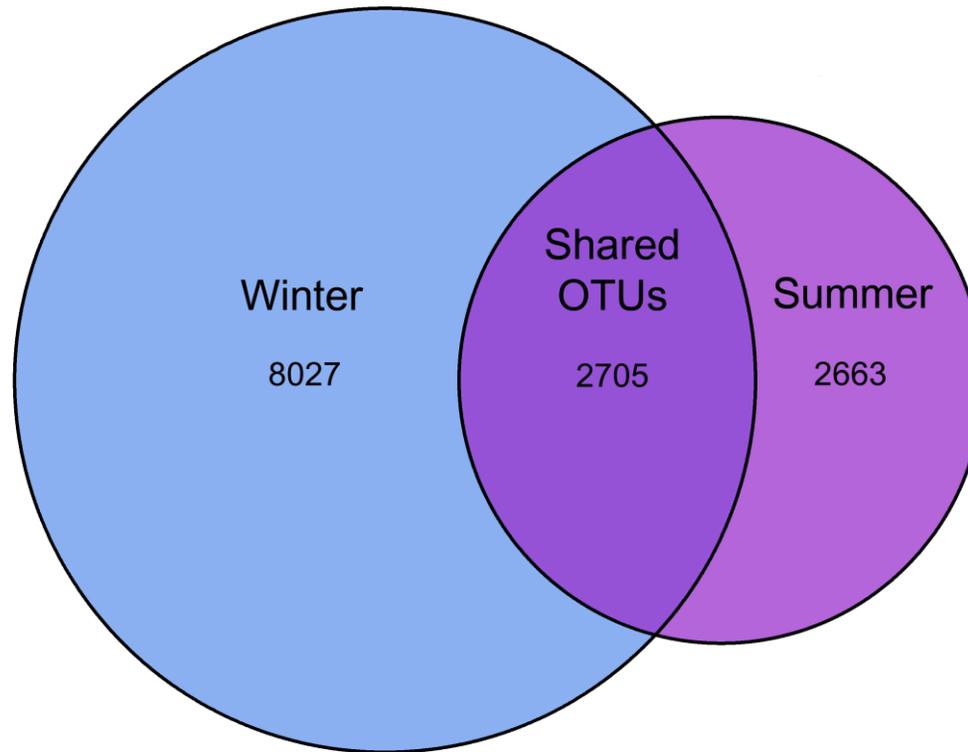
**Figure 7.** Rarefaction curves describing the bacterial (a) OTU richness and (b) phylogenetic diversity observed in the bioaerosol of SCBs during the winter and summer seasons. Diversity indices were calculated using random selections of 15,909 sequences per sample and error bars represent +1 s.e.m.

## **Effect of season and microclimatic parameters on community composition of bacterial bioaerosols**

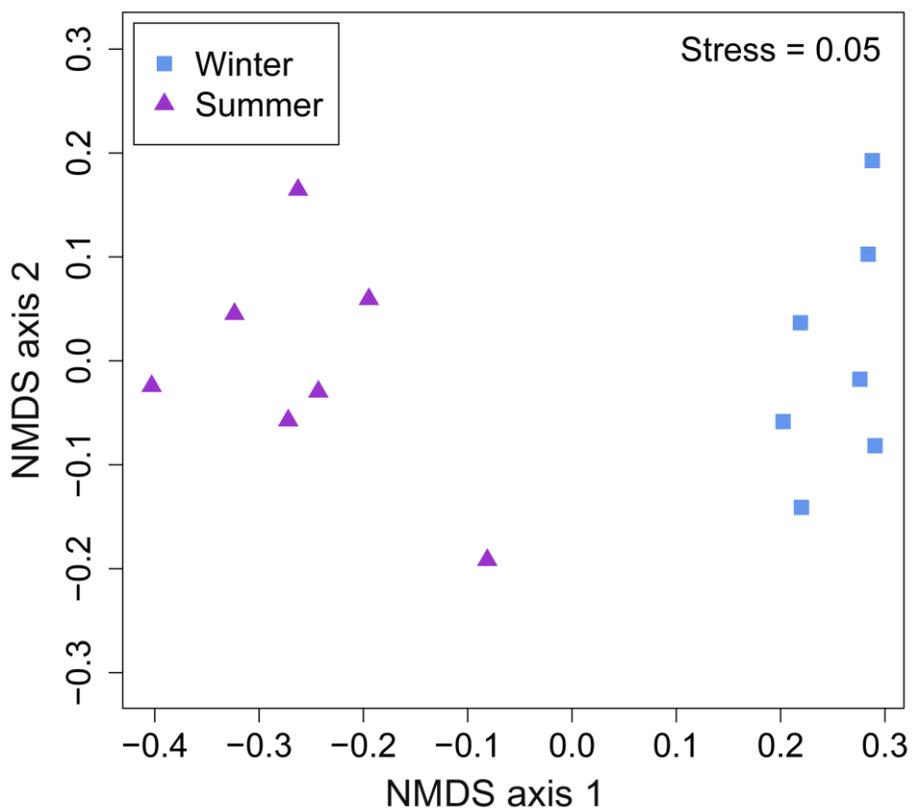
Venn diagrams (Figure 8) illustrate that OTU overlap between seasons as well as show unique OTUs. Overall, 20% of OTUs were shared between the winter and summer season. These 2,705 shared OTUs represented the majority of sequences (457,100 sequences or 91% of the total data set) (Figure 8). The OTU composition of the airborne bacterial communities was significantly influenced by seasons (ANOSIM statistic  $R = 0.96$ ,  $P < 0.01$ ; Figure 9). The samples collected during the winter season harbored bacterial communities distinct from those found in samples collected during summer. An environmental fitting analysis, using microclimatic variables, showed that airspeed ( $r^2 = 0.70$ ,  $P = 0.002$ ), PM<sub>2.5</sub> ( $r^2 = 0.39$ ,  $P = 0.04$ ), TSP ( $r^2 = 0.39$ ,  $P = 0.04$ ) and CO<sub>2</sub> ( $r^2 = 0.43$ ,  $P = 0.04$ ) were significantly associated with bacterial community composition. In several previous studies, it has been reported that microclimate variables are the most important factor which affects the indoor bacterial bioaerosol community composition and diversity (Kembel *et al.* 2012; Kembel *et al.* 2014). This relationship could be due to a direct link between the growth and survival of certain taxa and microclimate conditions in SCBs, or an increase in the dispersal of microbes from animals or animal feces under these conditions.

The most abundant bacterial phyla were *Firmicutes*, representing 50.9% of all sequences, followed by *Bacteroidetes* (21.3%), *Proteobacteria* (18.5%), and, to a lesser degree, *Actinobacteria* (3.9%), *Tenericutes* (0.6%), and *Spirochaetes* (0.4%); 0.8% of all sequences were unclassified. The phylum distribution

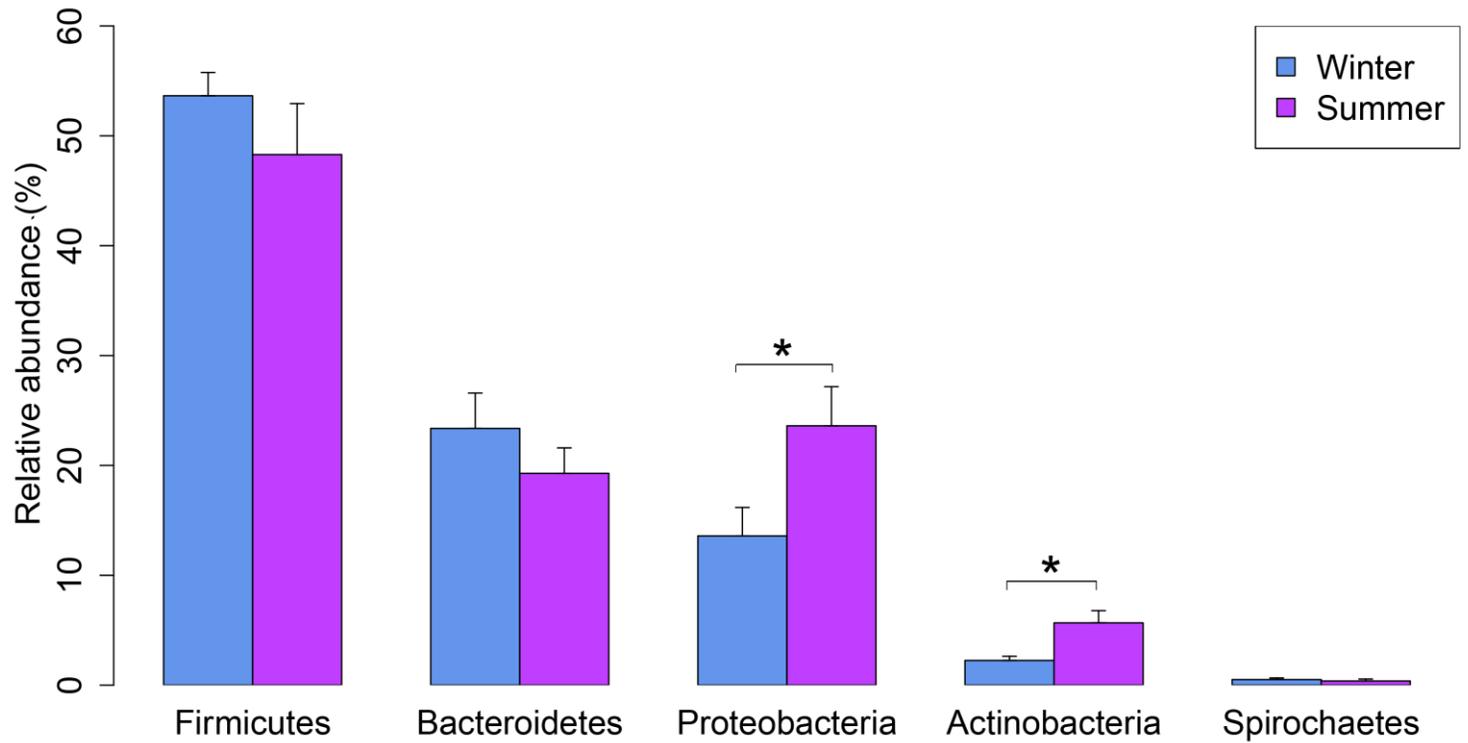
observed in this study in bioaerosol samples of SCBs is consistent with previous studies (Hong *et al.* 2012; Kristiansen *et al.* 2012; Nehme *et al.* 2008). We found significant differences in relative abundance across seasons for *Proteobacteria* ( $P = 0.04$ ) (Figure 10) and *Actinobacteria* ( $P = 0.03$ ) (Figure 10). We investigated in more detail what bacterial genera determine more strongly the distinct community composition in each season. Some bacterial genera were found to be dominant in both winter and summer seasons. *Lactobacillus* (18.3% and 16.3% on average) and *Prevotella* (19.6% and 6.2%) were the most dominant genera in both seasons. There were also some differences in abundant genera between the two seasons (Table 7). The bacterial bioaerosol of SCB in the winter season was dominated by a single genus – *Prevotella* – at nearly 19.6% (Metastats  $P = 0.01$ ). *Faecalibacterium* (0.8%), *Blutia* (0.7%), and *Catenibacterium* (0.7%) were also more abundant in the winter than in the summer (all  $P \leq 0.01$ ). Genera that were more abundant in the summer included *Sphingomonas* (3.7%), *Capnocytophaga* (3%), *Haemophilus* (2.8%), and *Streptococcus* (2.6%) (all  $P \leq 0.01$ ). The seasonal difference in the relative abundance of several bacterial genera justifying the view that the bacterial bioaerosol communities in both the winter and summer season samples are different.



**Figure 8.** Venn diagrams showing the overlap of OTUs (at 97% similarity) between winter and summer seasons. All samples in each season were pooled and then the percentage of shared and season-specific OTUs was calculated.



**Figure 9.** NMDS of Bray-Curtis pairwise dissimilarity of bacterial bioaerosol community in SCBs during the winter and summer seasons.



**Figure 10.** Relative abundance (means ± SE) of the most abundant bacterial phyla detected in SCBs bioaerosol during the winter and summer seasons. \* indicates significantly different at .05.

**Table 7.** Differentially abundant bacterial genera in swine confinement buildings sampled during winter and summer seasons. Differences in relative abundance of bacterial genera between seasons are represented with Metastats p-values. Significantly different ( $P \leq 0.01$ ) and relatively abundant genera ( $> 0.3\%$ ) were displayed.

<b>Phylum</b>	<b>Genus</b>	<b>Winter (%)</b>	<b>Summer (%)</b>	<b>P-value</b>
<i>Bacteroidetes</i>	<i>Capnocytophaga</i>	0.00	<b>3.06</b>	< 0.01
<i>Bacteroidetes</i>	<i>Chitinophaga</i>	0.01	<b>0.68</b>	< 0.01
<i>Bacteroidetes</i>	<i>Dyadobacter</i>	0.00	<b>0.37</b>	< 0.01
<i>Bacteroidetes</i>	<i>Prevotella</i>	<b>19.57</b>	6.24	0.01
<i>Firmicutes</i>	<i>Blautia</i>	<b>0.75</b>	0.19	0.01
<i>Firmicutes</i>	<i>Bulleidia</i>	<b>0.56</b>	0.33	< 0.01
<i>Firmicutes</i>	<i>Butyricicoccus</i>	<b>0.49</b>	0.19	0.01
<i>Firmicutes</i>	<i>Catenibacterium</i>	<b>0.74</b>	0.28	< 0.01
<i>Firmicutes</i>	<i>Faecalibacterium</i>	<b>0.87</b>	0.20	< 0.01
<i>Firmicutes</i>	<i>Oscillibacter</i>	<b>0.38</b>	0.12	0.01
<i>Firmicutes</i>	<i>Ruminococcus</i>	<b>0.33</b>	0.11	< 0.01
<i>Firmicutes</i>	<i>Streptococcus</i>	0.37	<b>2.64</b>	0.01
<i>Firmicutes</i>	<i>Subdoligranulum</i>	<b>0.69</b>	0.28	0.01
<i>Proteobacteria</i>	<i>Escherichia</i>	0.02	<b>1.26</b>	< 0.01
<i>Proteobacteria</i>	<i>Haemophilus</i>	0.00	<b>2.82</b>	< 0.01
<i>Proteobacteria</i>	<i>Neisseria</i>	0.00	<b>0.32</b>	0.01
<i>Proteobacteria</i>	<i>Sphingomonas</i>	0.17	<b>3.70</b>	0.01

## Comparison of aerial and fecal bacterial community

The bacterial bioaerosol community was compared with fecal bacterial community to see the similarity and differences in aerial and fecal bacterial community. At the phylum level both aerosol and fecal samples had very similar composition (Figure 11a). There were some differences in relative abundance of dominant bacterial genera in aerial and fecal samples (Figure 11b), however these dominant genera were present in both aerial and fecal samples (Figure 11b). Venn diagram was used to illustrate the overlap of bacterial OTUs between aerial and fecal samples. Overall, 39% of bacterial OTUs were shared between the aerial and fecal samples. These 1,242 shared bacteria OTUs represented the majority of sequences (90.8% of the total sequences) (Figure 12). These results validate the previous claims that swine feces are the primary source of the bacteria in the bioaerosols of SCBs (Hong *et al.* 2012; Nehme *et al.* 2008; Nonnenmann *et al.* 2010).

## Seasonal variations in abundance of 16S rRNA and Tc<sup>R</sup> genes

The abundances of 16S rRNA genes in the bioaerosols of SCBs were significantly higher in the winter (mean =  $1.4 \times 10^8$  bacteria  $m^{-3}$ ,  $n = 7$ ,  $P < 0.01$ , Figure 13) than in the summer (mean =  $1.2 \times 10^7$  bacteria  $m^{-3}$ ,  $P < 0.01$ ,  $n = 7$ , Figure 13). Six classes of Tc<sup>R</sup> genes (*tetB*, *tetH*, *tetZ*, *tetO*, *tetQ*, and *tetW*) were further quantified using qPCR. All six classes of Tc<sup>R</sup> genes were detected in high abundance in both winter and summer bioaerosol samples (Figure 13). Tc<sup>R</sup> genes encoding RPP (*tetO*, *tetQ* and *tetW*) were present in significantly higher copy numbers than Tc<sup>R</sup> encoding EFP (*tetB*, *tetH* and *tetZ*; *t*-test,  $P$ -value = 0.04). Seasonal trends were also detected in four Tc<sup>R</sup> genes,

which include *tetH*, *tetO*, *tetQ*, and *tetW*, and their abundances peaked in bioaerosol samples collected during the winter (Figure 13). The high abundance of 16S rRNA and Tc<sup>R</sup> genes detected in the bioaerosol samples suggests an alternative airborne transmission route, which can lead to their persistence and dispersal into the nearby environment. The level of contamination detected in this study was far higher than the proposed limit of bacterial contamination associated with human respiratory symptoms (Cormier *et al.* 1990). Similar to several previous studies, our results indicate that swine workers are exposed to a higher level of airborne bacteria than the occupational recommendations (Chang *et al.* 2001; Kristiansen *et al.* 2012; Nehme *et al.* 2008). Seasonal trends in microbial 16S rRNA gene concentration have already been reported by Nehme *et al.* (2009; 2008), who showed that total microbial 16S rRNA genes concentrations in bioaerosol of SCBs were significantly higher in winter. Seasonal fluctuation in Tc<sup>R</sup> genes abundances have been reported several times in wastewater treatment plants and livestock lagoons (McKinney *et al.* 2010; Yang *et al.* 2013); however, this is the first time a seasonal trend has been reported in bioaerosol samples of SCBs.

The prevalence of Tc<sup>R</sup> genes encode RPP in the present study is not surprising, since these genes were found to be predominant in the gastrointestinal tracts of pigs and steers (Aminov *et al.* 2001), and their elevated possibilities of transfer from one bacteria to another because of their close relationship with mobile genetic elements such as plasmids, conjugative transposons, integrons, and consequently their wide host range (Chopra & Roberts 2001; Roberts *et al.* 2012). Among all Tc<sup>R</sup> genes, *tetQ* had the highest abundance in bioaerosol samples (the

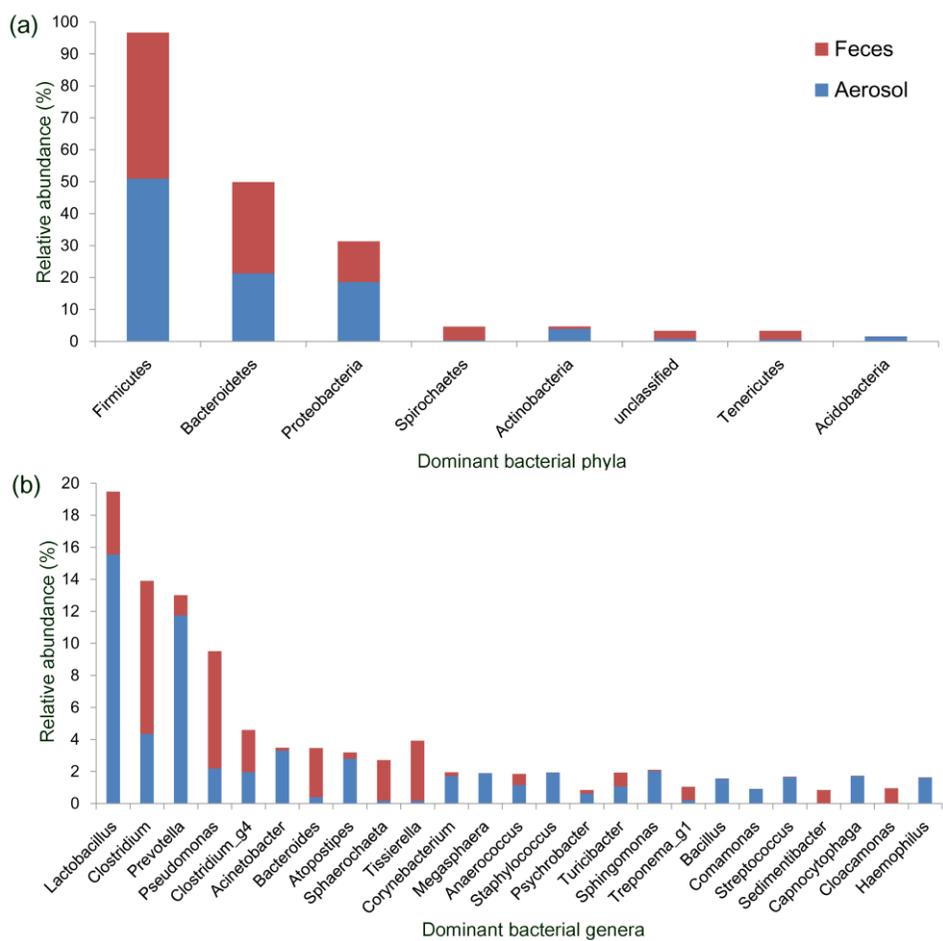
average abundance was  $8.89 \times 10^5 \pm 1.45 \times 10^6$  copies  $m^{-3}$ ) followed by *tetZ*, *tetO*, *tetW* and *tetH*, with *tetB* having the least abundance. The relatively high level of *tetQ* is not surprising because it is seen equally in both Gram-positive and Gram-negative bacterial genera (Roberts *et al.* 2012), and most of which have been shown to dominate the bioaerosols of SCBs such as *Clostridium*, *Lactobacillus*, *Staphylococcus*, *Streptococcus*, *Prevotella* etc. (Hong *et al.* 2012; Kristiansen *et al.* 2012; Nehme *et al.* 2008). The lack of *tetB* and *tetH* is also not surprising because they are found only associated with Gram-negative bacteria (Roberts *et al.* 2012), which are less common in bioaerosols of SCBs. However, higher average abundance of *tetZ* compared to *tetO* and *tetW* was not expected because it has relatively narrow host range (Only detected in *Lactobacillus* and *Corynebacterium*; (Roberts *et al.* 2012)). These results are consistent with the recent findings that both ecology and bacterial phylogeny are the primary determinant of ARG content in environment (Forsberg *et al.* 2014; Gibson *et al.* 2014). qPCR is more indicative of the potential for aerosol-mediated transfer of antibiotic resistance between environments than culture-based methods, and results can be more easily compared among studies. Nonetheless, the method is limited by its ability to detect only a fragment of genes targeted. Truncated sequences and non-expressed sequences cannot be resolved from expressed gene sequences using qPCR, so the levels reported could overestimate the number of functional, full length genes.

Among the six microclimate variables, air speed was found negatively correlated ( $P < 0.05$ ) with the abundances of 16S rRNA, *tetH*, *tetO*, *tetQ*, and *tetW* genes (Table 3), however, PM<sub>2.5</sub> and TSP

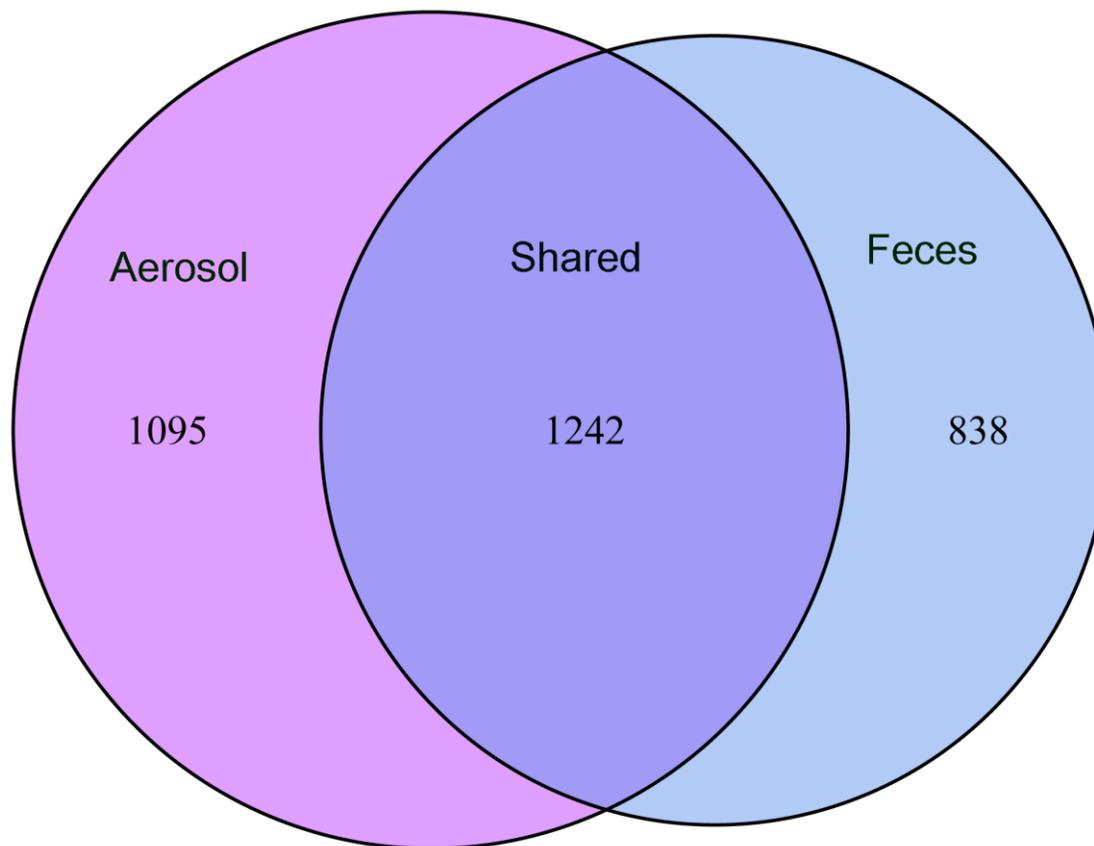
were found positively correlated ( $P < 0.05$ ) with these genes (Table 6). The reduced ventilation in SCBs to avoid heat loss in winter could be responsible for the increased concentration of TSP and PM<sub>2.5</sub> in SCBs, which in turn could increase the abundance of 16S rRNA and Tc<sup>R</sup> genes. In this survey only a limited number of ARGs were investigated in bioaerosols, however, a variety of ARGs encoding different antibiotic resistance could be present in bioaerosols of SCBs, where different classes of antibiotics (e.g., macrolides, lincosamides) are frequently used in addition to tetracycline. So there is a need for further study to explore more diverse ARGs in bioaerosols of SCBs.

The detection of 16S rRNA and Tc<sup>R</sup> genes in high abundance is of particular concern, because Tc<sup>R</sup> airborne pathogenic bacteria present in SCBs have been shown to colonize the nasal flora of pig farmers (Létourneau *et al.* 2010), and this could pose potential occupational health problem. Indoor air ventilated from the SCBs to the external environment can cause detrimental effects to the ambient air quality. For instance, multiple drug resistances bacteria were recovered in an air plume up to 150 m downwind from a SCB at higher percentages than upwind (Gibbs *et al.* 2006). Furthermore, presence of some airborne pathogens have been detected over long distances from their emission site which were found capable to infect healthy animals intramuscularly or intratracheally (Otake *et al.* 2010). Most of the previous studies consistently indicated an association between environmental exposure to SCBs and respiratory symptoms indicative of asthma of their neighbors (Kilburn 2012; Pavilonis *et al.* 2013; Schinasi *et al.* 2011). Surprisingly, Smit *et al.* (2013) found an inverse association between indicators of air pollution from livestock farms and

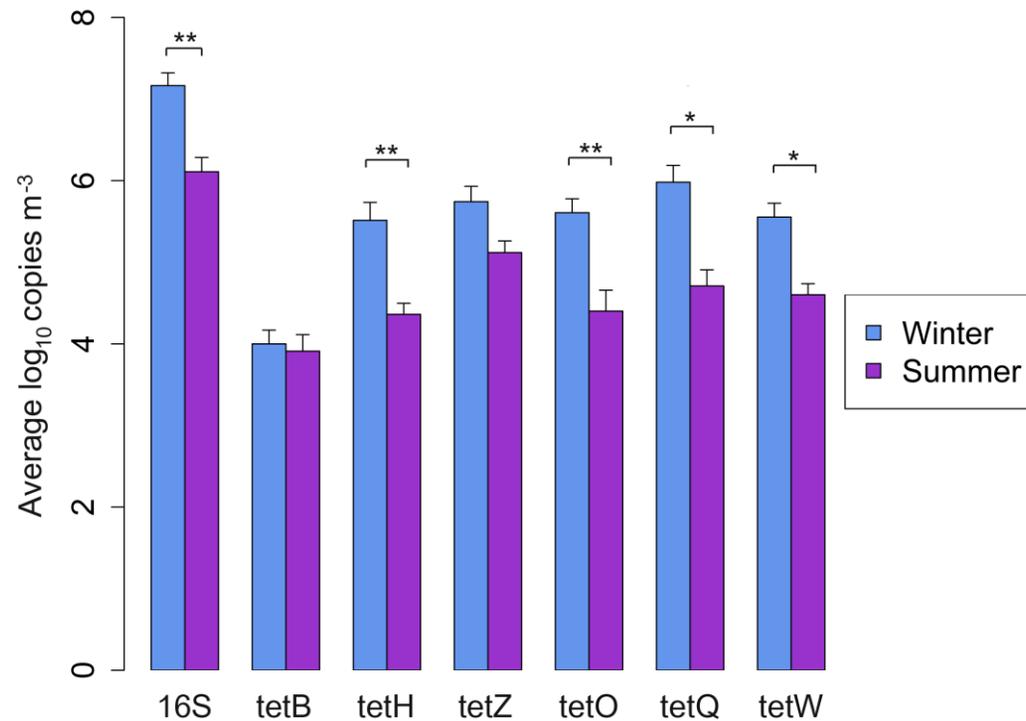
respiratory morbidity among neighboring resident. However, before drawing firm conclusions from this study, these results should be confirmed with more objective disease information.



**Figure 11.** Comparison of relative abundance of (a) dominant bacterial phyla and (b) dominant bacterial genera between aerial and fecal samples.



**Figure 12.** Venn diagrams showing the overlap of bacterial OTUs between aerosol and fecal samples.



**Figure 13.** Abundance of 16S rRNA and tetracycline resistance genes in the bioaerosols of SCBs. Asterisks above solid lines indicate significant differences between the winter and summer seasons samples of SCBs. \* indicates significantly different at  $< 0.05$ , \*\* indicates at  $< 0.01$ .

### **3.1.4. Conclusions**

Though, this study had fewer samples than previous studies, our results however indicate that seasons have an influence on the biotic contaminants abundance, community composition and diversity, in indoor air of SCBs. Seasonality was significantly associated with microclimate variables, indicating that indoor environmental conditions play an important role in structuring airborne biotic contaminants in SCBs. Based on the results of this study, better management practices and regulations can be designed to minimize the potential health impact on both the farm workers and the public residing in close proximity to these buildings.

## **3.2. Seasonal variations in community composition and the diversity of airborne fungi in swine confinement buildings**

### **3.2.1. Introduction**

Modern animal husbandry has changed in recent years from pasture-based animal farming to the use of confinement buildings with high animal density. The concentrations of volatile organic compounds, ammonia (NH<sub>3</sub>), sulfide and particulate matter are elevated in the indoor environment of confinement buildings due to the high animal density (Cambra-López *et al.* 2010; O'Neill & Phillips 1992), which leads to poor indoor air quality. The particulate matter contains fungal spores and endotoxins, which can cause lung infections and airway-related inflammatory responses in both farmers and animals (Auvermann *et al.* 2006; Bakutis *et al.* 2004; Radon *et al.* 2001).

Culture-based methods have been predominantly used in earlier studies of airborne fungi in various animal confinement buildings (Chang *et al.* 2001; Clark *et al.* 1983; Predicala *et al.* 2002). The fungal colony forming units (cfu) reported in these studies range in concentration from 10<sup>3</sup> cfu/m<sup>3</sup> to 10<sup>6</sup> cfu/m<sup>3</sup>, and *Cladosporium*, *Aspergillus* and *Penicillium* were detected as the predominant fungal genera. Other genera were detected, including *Alternaria*, *Fusarium*, *Verticillium*, and *Geotrichum*. It has also been found that indoor air fungal concentrations and emissions are influenced by the manure removal system in SCBs (Kim *et al.* 2008c). Viegas *et al.* (2013) studied air borne fungi in Portuguese swine farms and detected keratinophilic (*Scopulariopsis brevicaulis*) and toxigenic fungi

(*Aspergillus*, *Fusarium*, and *Penicillium* genera and *Stachybotrys chartarum*), suggesting a potential occupational health threat. Jo et al. (2005) studied airborne fungi concentrations in swine sheds and reported that the summer concentrations of total fungi and fungal genera inside the swine sheds were substantially higher than the winter values. A recent study of the fungal community of swine confinement facility aerosols using amplification of small subunit rRNA found *Aspergillus-Eurotium* as the quantitatively most important fungal group (Kristiansen *et al.* 2012). In an another recent study, Boissy et al. (2014) used shotgun pyrosequencing metagenomic analyses of DNA from settled surface dusts from swine confinement facilities and grain elevators and found that the fungal species DNA reads were 30-fold lower in swine confinement facilities than in grain elevators. However, to the best of our knowledge, no molecular studies have investigated the seasonal trends of airborne fungal community composition and diversity in SCBs.

In this study, we collected aerosol samples from seven commercial swine farms in South Korea during the winter and summer seasons, and the fungal community composition and diversity were analyzed using the Illumina Hiseq sequencing platform. The objectives of this study were as follows:

(1) To determine the effect of season on airborne fungal community composition and diversity in SCBs.

(2) To identify the potential human allergen/pathogen related fungal genera present in SCBs.

(3) To study how their relative abundances vary between the winter and summer seasons.

### **3.2.2. Materials and methods**

#### **Aerosol collection**

Aerosol samples were collected from seven commercial swine farms in South Korea in winter (January) and summer (June) of 2013, with prior permissions from farm owners. Detailed characteristics of SCBs and the methods used to collect aerosol samples have been described in section 3.1.2. We collected aerosol samples at a height of 1.4 m above the ground from three points in SCBs. Sterile cellulose nitrate filters (0.22  $\mu\text{m}$ ; Fisher Scientific, Pittsburgh, PA) were used to collect the aerosol samples via filtration with a flow rate of 4 L  $\text{min}^{-1}$  for 24 h. After aerosol collection, the filters were immediately maintained at 4 °C until transport to the laboratory, where the samples were frozen at -20 °C.

#### **DNA extraction and PCR amplification**

The PowerSoil DNA isolation kit (MoBio Laboratories, Carlsbad, CA) was used to extract DNA from filters by following the initial processing methods as described in Kumari et al (2014). The purified DNA samples were stored at -20 °C until PCR amplification. We amplified the ITS1 region using primer pairs ITS1FI2, 5'-GAACCGCGGARGGATCA-3' (Schmidt *et al.* 2013) and ITS2, 5'-GAACCGCGGARGGATCA-3' (White *et al.* 1990).

#### **Sequencing and data processing**

The amplicons were sequenced at the Beijing Genome Institute (BGI) (Hong Kong, China) using 2 $\times$ 150 bp Hiseq2000 (Illumina, San

Diego, CA, USA) according to the manufacturer's instructions. Pandaseq software was used to assemble the paired-end sequences with default quality score value, i.e., 0.6 (Masella *et al.* 2012). Furthermore, ambiguous bases and homopolymers (> 8 bp) were removed from assembled sequences using mothur (Schloss *et al.* 2009). Chimeric sequences were removed using the 'chimera.uchime' command in mothur (Edgar *et al.* 2011). To remove the sampling bias, we used the 'sub.sample' command in mothur to randomly select a subset of 24,000 sequences from each. The standardized sequences were classified against a named fungal ITS sequences database (Nilsson *et al.* 2009) using BLASTn ver. 2.2.19 (Altschul *et al.* 1990). Next, a fungal taxonomic identification tool FHiTINGS (Dannemiller *et al.* 2014c) was used with BLASTn output files to sum and sort the taxonomic ranks from kingdom to species. The potential human allergen/pathogen related fungal taxa were identified using a list of known fungal allergen/pathogen related taxa (Yamamoto *et al.* 2012). Furthermore, the QIIME implementation of UCLUST (Edgar 2010) was used to assign operational taxonomic units (OTUs) with a threshold of 97% sequence similarity. All sequence data are deposited in the MG-RAST server (Meyer *et al.* 2008) under MG-RAST IDs 4633146.3–4633187.3.

### **Statistical analysis**

The seasonal differences in OTU richness, Shannon index and the relative abundance of the most abundant phyla were evaluated using t-test and Wilcoxon rank-sum test for normal and non-normal data, respectively. Differentially abundant fungal classes and genera between the winter and summer seasons were identified using a

parametric approach (Metastats) (White *et al.* 2009). The p-values were adjusted using Benjamini and Hochberg methods to account for multiple comparisons (Benjamini & Hochberg 1995). Adjusted p-values of less than 0.05 were considered statistically significant. The co-occurrence network of all of the detected fungal genera was constructed using CONET software by following the example tutorial (<http://psbweb05.psb.ugent.be/conet/microbialnetworks/conet.php>) (Faust *et al.* 2012) and visualized using Cytoscape (Smoot *et al.* 2011). The Bray-Curtis distance was used to calculate the OTU-based community dissimilarity (Magurran 2013). Non-metric multidimensional scaling (NMDS) was used to visualize the change in OTU composition across the seasons. The seasonal difference in community composition was tested using an analysis of similarity (ANOSIM) in the vegan R package (Oksanen *et al.* 2007). We also performed a permutational dispersion analysis to test whether beta diversity is significantly different between seasons by using the betadisper function in the vegan R package (Anderson 2006). All statistical analysis, graphs, and ordinations were produced using R version 3.0.2 (RDevelopmentCoreTeam 2008).

### **3.2.3. Results and discussion**

In this study, we used a high-throughput Illumina Miseq platform to extensively study the airborne fungal community composition and diversity in SCBs. While several studies have comprehensively investigated the airborne bacterial community composition and diversity in SCBs using next-generation sequencing (NGS) methods (Hong *et al.* 2012; Kumari & Choi 2014), relatively

little is known about the fungal community composition and diversity.

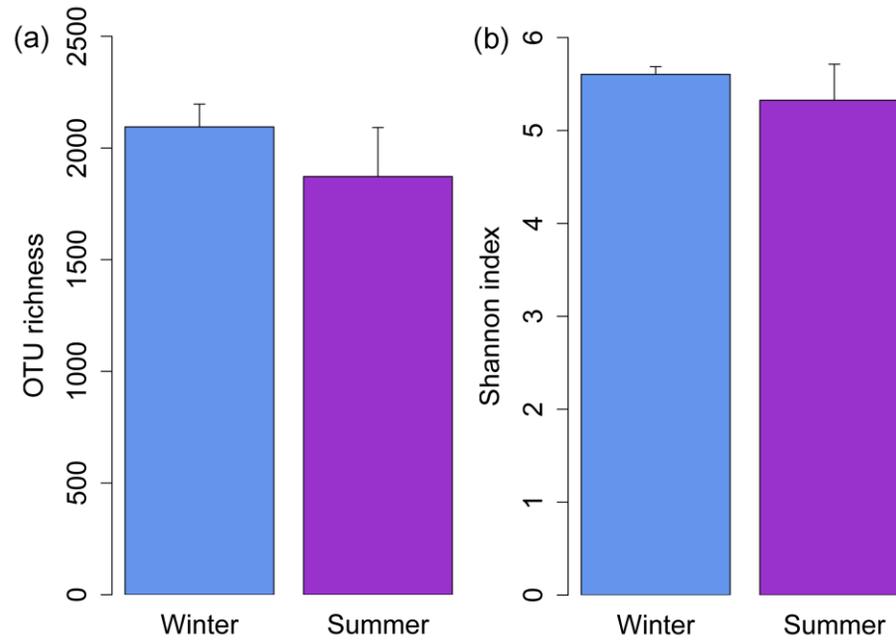
### **Effect of seasonal variations on fungal diversity**

From the 42 samples, we observed 22,399 OTUs at 97% sequence similarity from 1,008,000 good-quality sequences (24,000 randomly selected sequences per sample). The average number of the observed OTUs per sample was  $1,983 \pm 202$  (standard deviation [SD]), ranging from 1,524 to 2,345 OTUs. Similar to the bacterial diversity reported earlier in the same SCBs (Kumari & Choi 2014), the airborne fungal OTU richness and diversity were significantly higher in winter than in summer (Figure 14). Adams et al. (2013) also found higher fungal diversity in winter while studying the indoor air fungal community of residence buildings. One of the possible explanations of this high diversity could be that to maintain the indoor air temperature during winter, all of the openings are closed and the ventilation rates are reduced to minimal, which in turn increases the indoor bioaerosol particles and thus increases microbial diversity. The fungal richness and diversity information might be useful in terms of health and exposure evaluations of farmers working in SCBs, as fungal richness and diversity have been shown to be associated with asthma development (Dannemiller *et al.* 2014a; Ege *et al.* 2011).

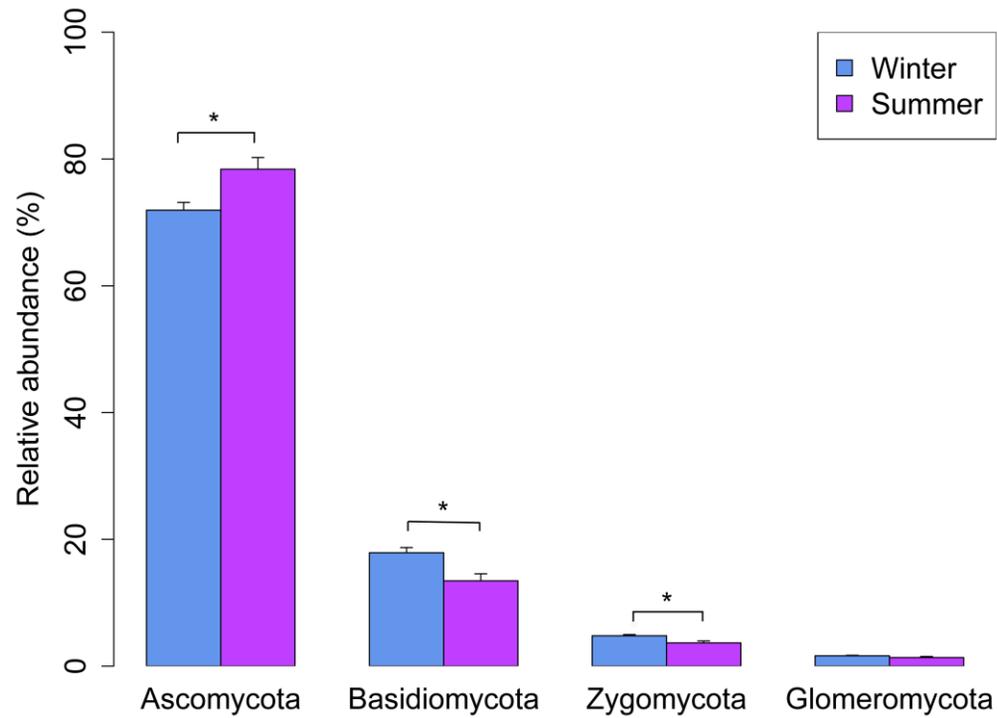
### **Effect of seasonal variations on fungal community composition**

The most abundant fungal phyla across all of samples were *Ascomycota*, representing 75.4% of all sequences, followed by *Basidiomycota* (15.3%), *Zygomycota* (4.2%), and *Glomeromycota* (1.5%) (Figure 15). The predominant fungal classes

detected in this study were *Dothideomycetes* and *Sordariomycetes* of phylum *Ascomycota*, which has been shown to dominate aerosol samples in several previous studies (Dannemiller *et al.* 2014b; Fröhlich-Nowoisky *et al.* 2009; Yamamoto *et al.* 2012). *Dothideomycetes* class is known to contain several allergenic fungal taxa (D'amato *et al.* 1997; Halonen *et al.* 1997; Shelton *et al.* 2002). *Agaricomycetes* was the most abundant class of phylum *Basidiomycota*, which does not generally contain described human allergen/pathogen related fungal taxa. The relative abundance of *Ascomycota* was significantly higher in summer ( $W=155$ ,  $P < 0.01$ ; Figure 15), whereas the relative abundances of *Basidiomycota* and *Zygomycota* were significantly higher in winter ( $W = 439$ ,  $P = 0.01$ ; Fig. 2). Comparisons between seasons were also conducted at the class and genus levels. After adjusting for multiple comparisons, the relative abundances of many classes and genera were significantly more abundant in a particular season (Table 8). The airborne fungal network contained 185 nodes (genera) and 1553 edges (lines connecting nodes) (Figure 16). Of these, 179 nodes and 1260 edges were positively associated, which means that they occur together and hence might share certain properties. Genus *Clavaria* was found to be connected to most of the positive edges (46 edges), whereas *Tomentella* connected to most of the negative edges (26 edges). The co-occurrence network analysis allowed us to identify the important relationships among fungal genera, which could be tested in the future under controlled conditions.



**Figure 14.** Seasonal variation in the fungal (a) OTU richness and (b) Shannon diversity index in swine confinement buildings.

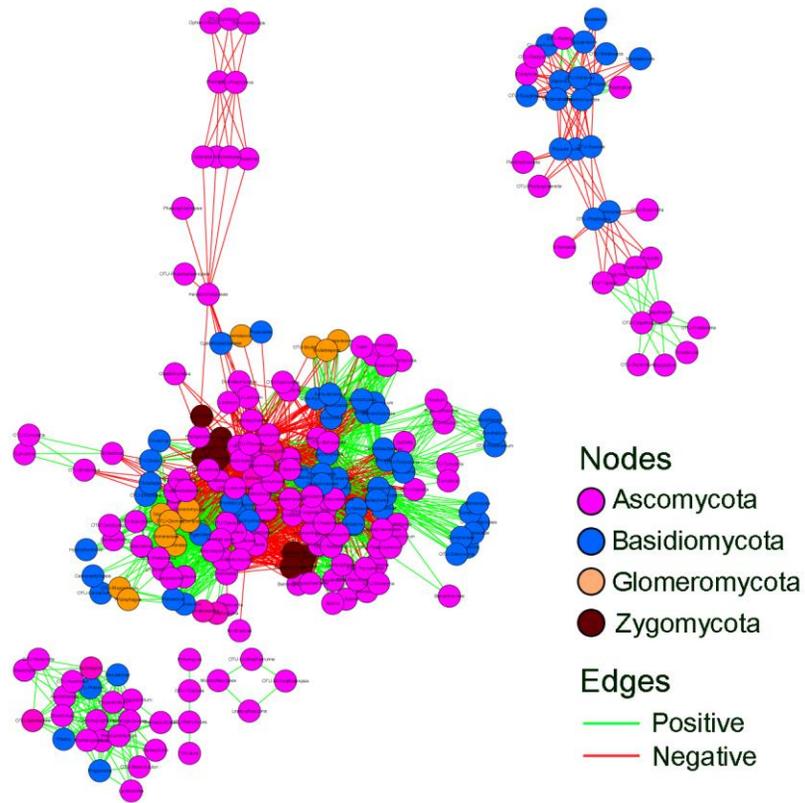


**Figure 15.** The relative abundance (means  $\pm$  SD) of the predominant fungal phyla of SCBs during the winter and summer seasons. The asterisk mark indicates significant differences between seasons.

**Table 8.** Differentially abundant fungal taxa in winter and summer seasons. The fungal taxa whose relative abundance was less than 0.01% across all samples were excluded. Only significantly different taxa ( $q < 0.05$ ) are displayed.

<b>Season</b>	<b>Class</b>	<b>Genus</b>
Winter	<i>Agaricomycetes</i>	<i>Trichosporon</i>
	<i>Pezizomycetes</i>	<i>Emericella</i>
	<i>Tremellomycetes</i>	<i>Calyptrozyma</i>
	<i>Saccharomycetes</i>	<i>Lophiostoma</i>
	<i>Pucciniomycetes</i>	<i>Devriesia</i>
		<i>Massarina</i>
		<i>Candida</i>
		<i>Teratosphaeria</i>
		<i>Ramariopsis</i>
		<i>Fimetariella</i>
		<i>Leptodontidium</i>
		<i>Sorocybe</i>
		<i>Tetracladium</i>
		<i>Knufia</i>
		<i>Cortinarius</i>
		<i>Scytalidium</i>
		<i>Marchandiobasidium</i>
		<i>Trametes</i>
		<i>Helvella</i>
		<i>Russula</i>
	<i>Trapelia</i>	
	<i>Mycosphaerella</i>	
	<i>Arnium</i>	
	<i>Loreleia</i>	
	<i>Daedaleopsis</i>	
	<i>Issatchenkia</i>	
	<i>Capronia</i>	

		<i>Cystofilobasidium</i>
		<i>Leucosporidiella</i>
		<i>Tremelloscypha</i>
		<i>Anzina</i>
		<i>Botryosphaeria</i>
	<i>Eurotiomycetes</i>	<i>Petromyces</i>
Summer	<i>Wallemiomycetes</i>	<i>Eurotium</i>
		<i>Golovinomyces</i>

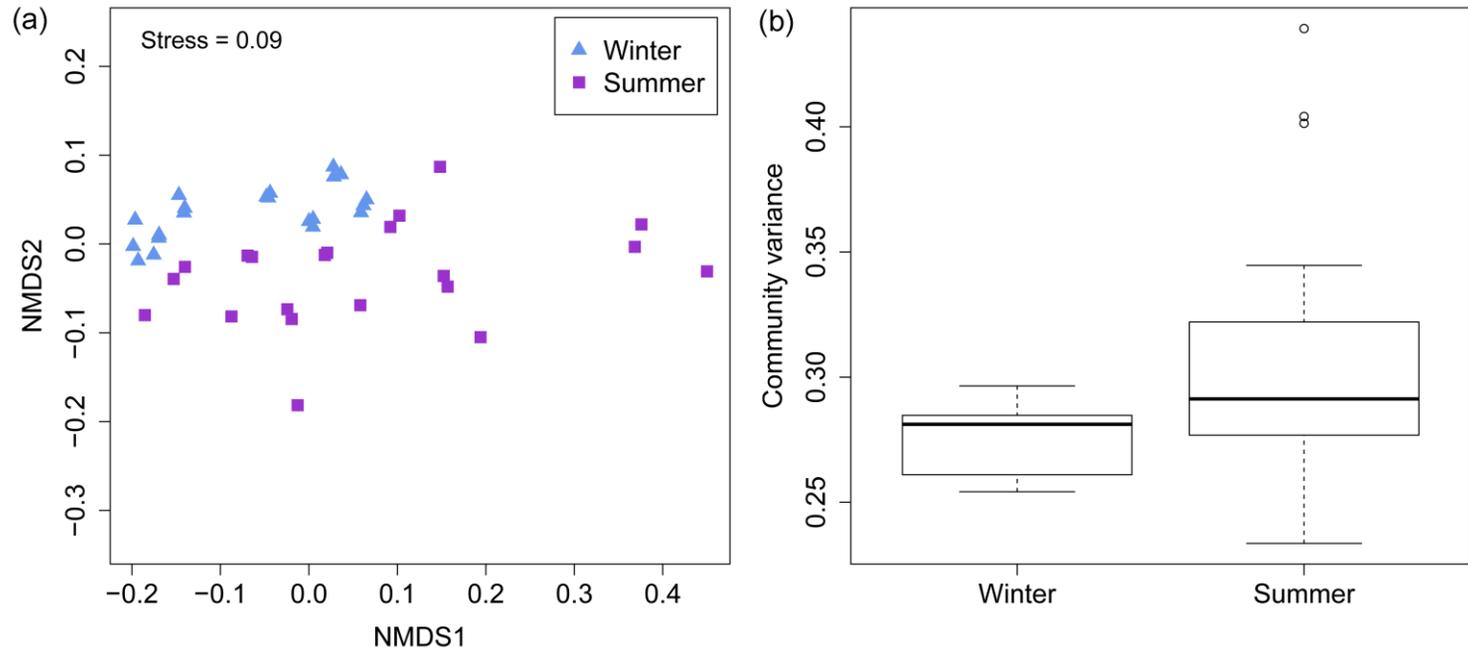


**Figure 16.** The co-occurrence network of air borne fungal communities in SCBs. Each node represents a fungal genus and lines connecting the nodes are edges.

An NMDS plot of Bray–Curtis distances showed that the composition of the airborne fungal communities was significantly influenced by seasons (ANOSIM statistic  $R = 0.96$ ,  $P < 0.01$ ; Figure 17a). Our results are consistent with the findings of several previous studies on indoor air fungi (Adams *et al.* 2013; Pitkäranta *et al.* 2008; Shelton *et al.* 2002). The beta diversity, measured as the average distance of all samples to the centroid within each season, varied significantly between seasons, with summer having significantly higher beta diversity than winter ( $P < 0.05$ ) (Figure 17b). These results suggest that the airborne fungal community composition is more heterogeneous in summer than in winter.

### **Potential human allergen/pathogen related fungal genera**

A total of 80 human allergen/pathogen related genera are known to date (Simon-Nobbe *et al.* 2008). Of these, we identified a total of 29 human allergen/pathogen related fungal genera in SCBs (Table 9). Overall, the average relative abundance of human allergen/pathogen related fungal genera was higher in winter than in summer (Figure 18). The most abundant human allergen/pathogen related fungal genus was *Fusarium* (10.8%). *Fusarium* is an emerging pathogen and can cause infections in humans, especially in immunocompromised hosts (Nucci & Anaissie 2002, 2007). Of the detected human allergen/pathogen related fungal genera, the relative abundances of four genera, namely, *Candida*, *Aspergillus*, *Pichia*, and *Trichosporon*, varied significantly between seasons, and except for *Aspergillus*, the relative abundances of the other three genera were significantly higher in winter.

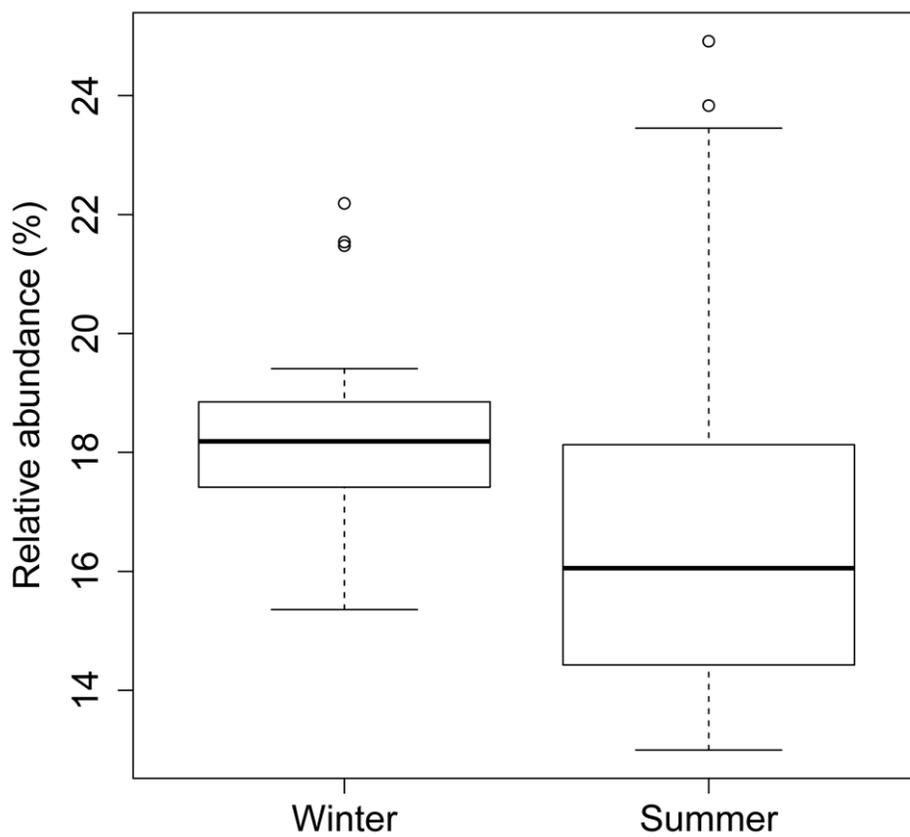


**Figure 17.** (a) NMDS plot showing fungal community composition of SCBs during the winter and summer seasons. (b) Community variance (beta diversity) of fungal community based on Bray-Curtis distance in the winter and summer seasons.

**Table 9.** Airborne fungal allergen/pathogen related genera identified in SCBs.

Name	<i>P</i> -value	Season
<i>Candida</i>	<b>0.01</b>	Winter
<i>Fusarium</i>	0.36	
<i>Rhodotorula</i>	0.22	
<i>Cryptococcus</i>	0.08	
<i>Aspergillus</i>	<b>0.01</b>	Summer
<i>Embellisia</i>	0.98	
<i>Alternaria</i>	0.11	
<i>Cladosporium</i>	0.05	
<i>Pichia</i>	<b>0.02</b>	Winter
<i>Trichosporon</i>	<b>0.01</b>	Winter
<i>Curvularia</i>	0.22	
<i>Penicillium</i>	0.90	
<i>Beauveria</i>	0.94	
<i>Ulocladium</i>	0.51	
<i>Pseudallescheria</i>	0.08	
<i>Sporothrix</i>	0.43	
<i>Chrysosporium</i>	0.11	
<i>Exophiala</i>	0.09	
<i>Stachybotrys</i>	0.22	
<i>Malassezia</i>	0.43	
<i>Epicoccum</i>	0.36	
<i>Nimbya</i>	0.98	
<i>Saccharomyces</i>	0.36	
<i>Pleospora</i>	1.00	
<i>Stemphylium</i>	0.22	
<i>Stemphylium</i>	0.22	
<i>Thermomyces</i>	0.22	
<i>Coprinus</i>	1.00	
<i>Psilocybe</i>	0.62	

All values with  $P < 0.05$  are in bold.



**Figure 18.** Box-plot showing the relative abundance of allergen/pathogen related fungal genera between winter and summer seasons in swine confinement buildings.

### **3.2.4. Conclusions**

In conclusion, through in-depth sequencing, we have shown that the airborne fungal community composition in SCBs was influenced by seasonal variations. Seasonality also influenced the alpha and beta diversities of airborne fungi in SCBs; however, both showed different patterns from one another, whereby alpha diversity peaked in winter and beta diversity peaked in summer. Several human allergen/pathogen related fungal genera were also detected in SCBs in both seasons, and in total, their relative abundance was higher in winter. These potential human allergen/pathogen related fungal genera present in the indoor air of SCBs may impact the health of farm workers, which suggests that better management practices are needed to minimize the risk of potential occupational health hazards in farm workers. However, it is to be noted that fungi are generally opportunistic pathogens, and most of the fungi detected in this study are common in the environment, so it is very unlikely that these opportunistic fungal pathogens can infect healthy individuals. Overall, this study provides a better understanding of seasonal patterns in the airborne fungal community composition and diversity in SCBs.

# **CHAPTER 4. MITIGATION OF AIBORNE CONTAMINANTS EMISSION FROM SWINE CONFINEMENT BUILDINGS**

# **Biofilter bacterial biofilm community succession reduces the emissions of airborne contaminants from swine confinement buildings**

## **4.1.1 Introduction**

The use of confinement swine buildings with high animal density has been intensified in recent years to meet the higher demand of meat products. These confinement structures emit various airborne contaminants, which can cause harmful effects to the public residing in close proximity to these buildings (detailed reviews on the airborne contaminants emitted from SCBs are mentioned in chapter 1). Therefore, reducing the emission of the airborne contaminants from SCBs is necessary in order to provide healthy environment to their neighbors.

Several mitigation strategies have been used earlier to reduce the emissions of airborne contaminants from SCBs (for detailed reviews please see the section 1.3), and it has been shown that biofilter is the most promising and cost-effective technology to reduce odorous gases emitted from livestock buildings (Estrada *et al.* 2012; Prado *et al.* 2009; Wani *et al.* 1997). Also, biofilter is an eco-friendly technology that uses no chemicals with potentially hazardous effects (Singh *et al.* 2006a; Singh *et al.* 2006b). Biofiltration is a complex process which involves interactions of absorption, adsorption, and biological degradation (Devanny *et al.* 1998). Though the performance of biofilters in reducing odorous gases were evaluated in several studies (Chen *et al.* 2008), little is known about the bacterial biofilm community immobilized in the packing material of biofilter which

helps in breaking down of the contaminants present in the air stream.

In the present study, we used a biological air filter to reduce the emissions of airborne contaminants from an experimental swine farm facility, and also investigated the successional development of bacterial biofilm community in the packing material of biofilter by using the Illumina Miseq sequencing platform. The term ‘biofilm’ being defined here as an adsorbed the thin- layered condensations of bacteria attached to the surface of cellulose pad filters. The objectives of this study were as follows:

(1) To evaluate the effect of biofilter on emission of airborne contaminants.

(2) To study the successional development of bacterial biofilm community in biofilter which helps in biological degradation of airborne contaminants present in the air stream.

## **4.1.2 Materials and methods**

### **Two-stage biofilter**

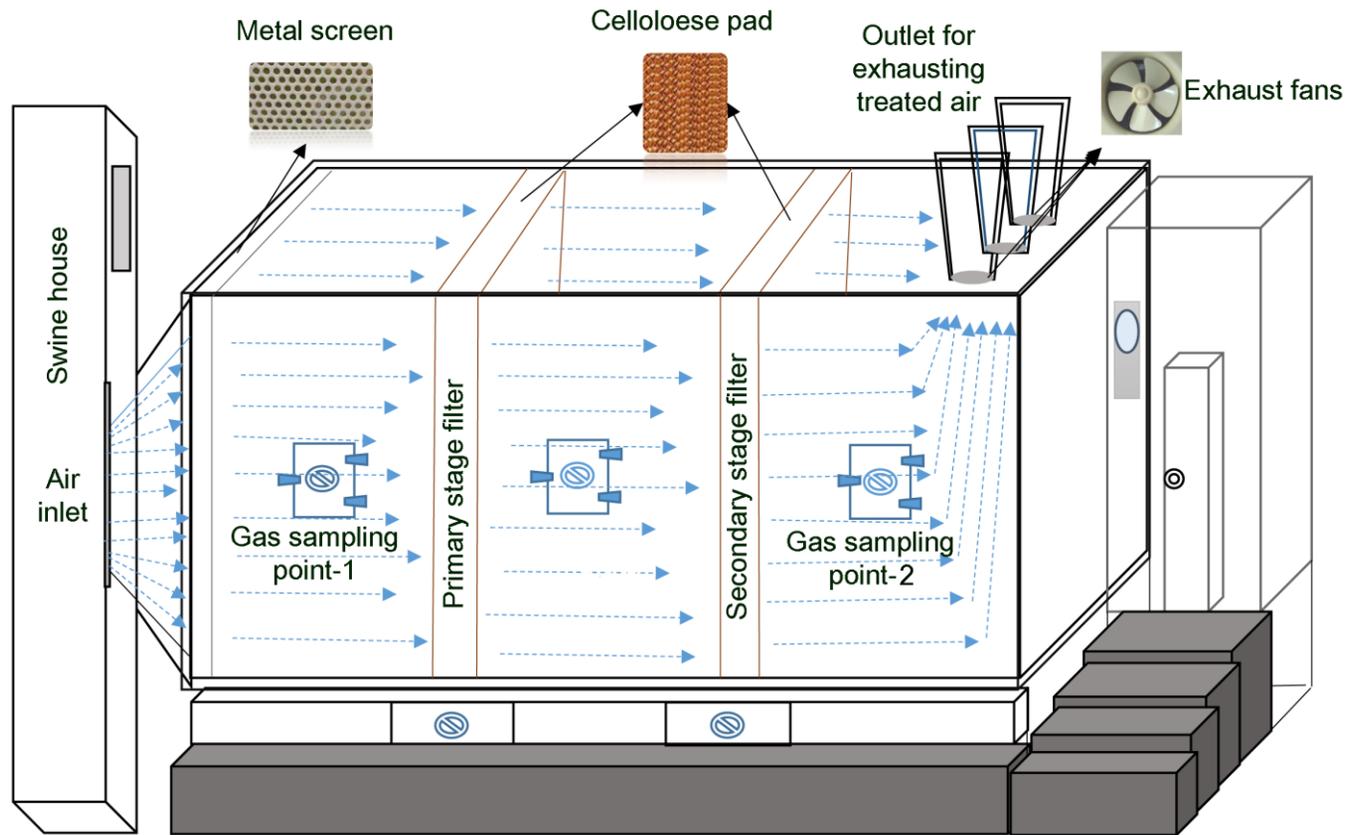
A two-stage biofilter (Figure A2) was installed next to an experimental swine farm facility (Figure A3) of Seoul National University located in Suwon, South Korea. There were 160 growing pigs were kept inside the experimental swine farm facility during the entire experiment period (11/02/2014–06/05/2014). The packing material used to design the biofilter was cellulose pads with two vertical filter walls (Figure 19). The depth of the both filters was 15 cm, and both of the filters were irrigated with recirculated water. The two-stage biofilter has designed to take up and metabolize different compounds at each stage. The first stage acts as a dust trap, where  $\text{NH}_3$ ,

dust particles and soluble organic compounds get dissolved into the water that is constantly irrigated over the filter surface. The second stage acts as a trickling biofilter, where irrigation is restricted to ensure better uptake and reduction of less soluble organic compounds by minimizing the liquid boundary layer on the surface of the biofilm. An air distribution screen was placed before first filter to ensure equal distribution of air in the biofilter and to lower the load of dust particles. The ventilation air was drawn through the biofilter from three outlets by using three ventilation fans which were placed after the biofilter.

### **Experimental setup and analysis of odorous gases**

The experiment was conducted for 12 weeks, and the reduction of odorous gases was measured every week. The odorous gases measured in this experiment were  $\text{NH}_3$ , acids-acetic acid, propionic acid, butyric acid, iso-butyric acid, valeric acid, methyl mercaptan, dimethyl sulphide, and dimethyl disulfide. Except  $\text{NH}_3$  all other odorous gases were analyzed by using gas chromatograph-mass spectrometer (Agilent GC6890N/5975C MS, Youngin, Korea) (Figure A4). For this analysis, the air samples were collected into a 1 L Tedlar bag (SKC Inc., Eighty-four, PA, USA) from the two sampling ports of the biofilter (Figure 19). After sampling, the bags were immediately transported to the laboratory and analyzed within 18 h by using solid-phase microextraction (SPME) fibers (Supelco, Bellefonte, PA, USA); the fiber type was 75-mm carboxen-polydimethylsiloxane. Samples were extracted by using SPME fibers for 30 min with a manual fibers holder from Supelco (Bellefonte, PA, USA). After extraction, the SPME fiber was removed from the Tedlar bag and immediately inserted

into the injection port of the GC-MS. The concentrations of  $\text{NH}_3$  were measure by using a GASTECH device (Pump kit No. 101).



**Figure 19.** Schematic diagram of the two-stage biofilter used in this study.

## **Sample collection and DNA extraction**

For analyzing the succession in bacterial biofilm community associated with the biofilter, samples were collected from both primary and secondary filters at days 14, 21, 35, 63, 84. The pieces of the cellulose filters were kept at 4°C until processing in the laboratory. Total DNA was extracted directly from the filters using the PowerSoil DNA isolation kit (MoBio Laboratories, Carlsbad, CA). The big pieces of the filters were aseptically cut into small pieces, loaded into the bead tube of the DNA extraction kit, and heated to 65°C for 10 min followed by 2 min of vortexing. The remaining steps of the DNA extraction were performed according to the manufacturer's instructions. The purified DNA was resuspended in 50 µl of solution S6 (MoBio Laboratories) and stored at -20°C until PCR amplification.

## **Illumina sequencing and data processing**

A single round of PCR is performed using "fusion primers" (Illumina adaptors + indices + specific regions) targeting the V6/V7/V8 regions of bacterial 16S rRNA genes (Comeau *et al.* 2011). The amplicons were sequenced at Centre for Comparative Genomics and Evolutionary Biology (CGEB), (Dalhousie University, Halifax, Canada) using paired-end (2×300 nt) Illumina sequencing with a MiSeq system (Illumina, USA). The mothur software package was used to process the sequence data (Schloss *et al.* 2009). First, paired-end sequence assembly was generated using the '*make.contigs*' command in mothur prior to quality trimming, sequence filtration and alignment against a SILVA alignment (<http://www.arb-silva.de/>). Next, the '*pre.cluster*' and '*chimera.uchime*' commands in mothur were used to

remove the sequencing errors and chimeric sequences, respectively (Edgar *et al.* 2011; Huse *et al.* 2010). Taxonomic annotations of all of the high quality sequences were obtained via '*classify.seq*' command in mothur using the reference Greengenes taxonomy database. A random subset of 17,852 sequences per sample was generated using the '*sub.sample*' command in mothur prior to statistical analysis. The bacterial operational taxonomic unit (OTU) matrix was built using '*dist.seqs*' command in mothur, and the generated distance matrix was used to cluster sequences into OTUs by mothur's '*cluster*' command using the average linkage algorithm. Finally, '*make.shared*' command was used to generate the bacterial OTUs at a cutoff value of 0.03, and the entire singleton OTUs were removed prior to analysis. The diversity indices were calculated using '*summary.single*' command in mothur.

### **Statistical processing and analysis of results**

The reduction efficiency of each odorous gas was determined using the relationships between the influent and effluent gas phase concentration, as follows:

$$\text{Reduction efficiency (\%)} = [(C_{\text{in}} - C_{\text{out}}) / C_{\text{in}}] \times 100$$

Where,

$C_{\text{in}}$  = Influent gas phase concentration

$C_{\text{out}}$  = Effluent gas phase concentration

The odorants reduction efficiencies of biofilter were correlated

to the sampling time using linear functions. The differences in bacterial community composition based on Bray-Curtis distances were visualized using non-metric multidimensional scaling (NMDS) plots. A permutational multivariate analysis of variance (PERMANOVA) was performed with 999 permutations using the Adonis function in VEGAN R package to test if bacterial biofilm communities of biofilter differed significantly by filtration stage and sampling time.

### **4.1.3 Results and discussion**

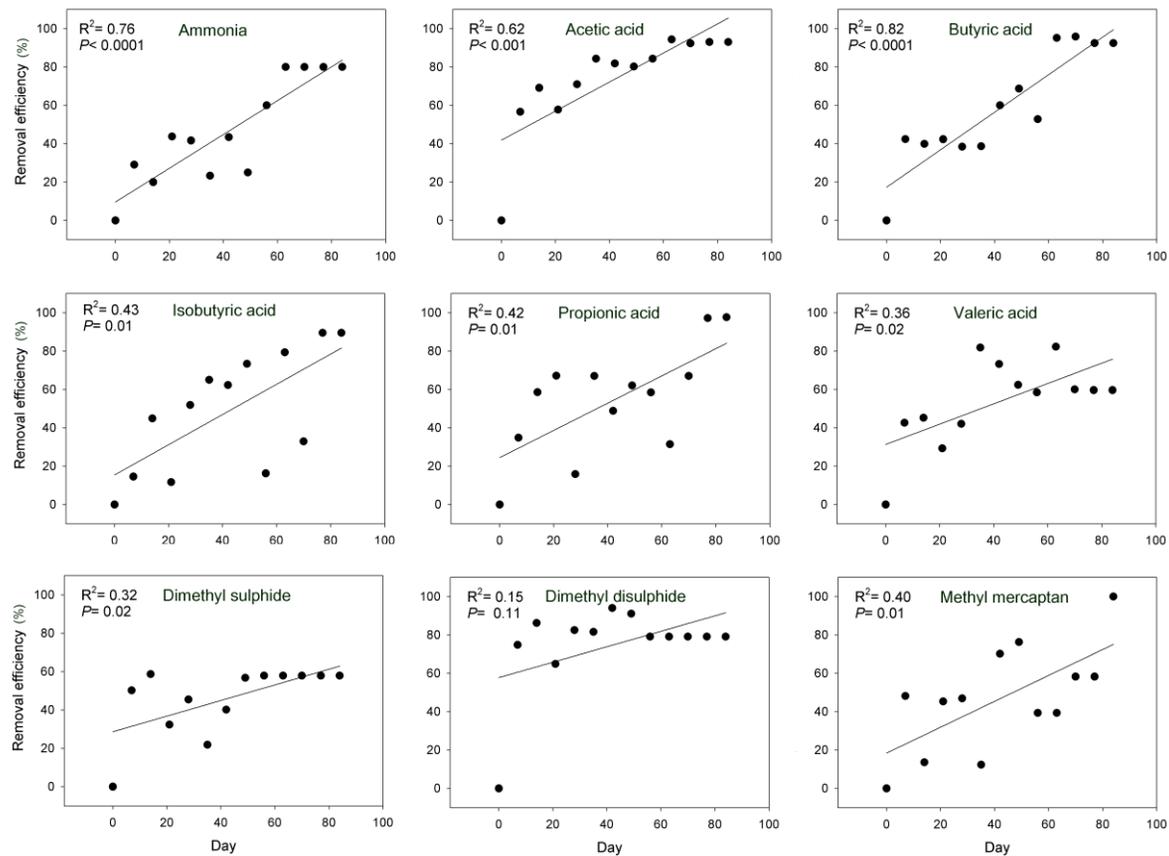
#### **Odor reduction efficiency of biofilter**

A total of nine odorous gases were measured during this experiment and the results indicate that all these odorants emissions were efficiency reduced by biofilter in efflux air from SCBs (Figure 20). The odorant reduction efficiency of biofilter was increased linearly with time (Figure 20). The reduction efficiency of methyl mercaptan was highest (100%) at day 84, whereas the reduction efficiency of dimethyl sulphide was lowest (57.9%) at day 84. These results are in agreement with other studies where biofiltration system was used to reduce the emissions of gaseous compounds from SCBs, and it has been shown that biofilter allows a 64–69% decrease in  $\text{NH}_3$  and an 85–92.5% reduction in other odorous gaseous compounds (Sheridan *et al.* 2002).

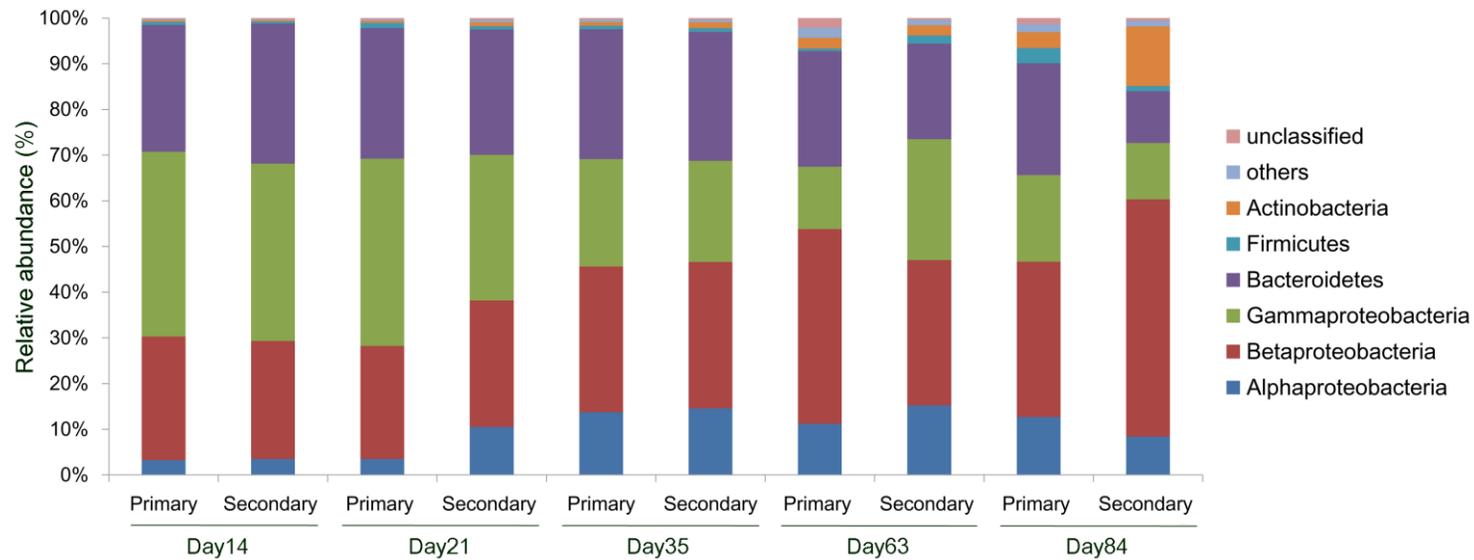
#### **Successional development of bacterial biofilm community**

The most abundant bacterial phyla across all samples were *Proteobacteria* (66.5%), followed by *Bacteroidetes* (22.9%) and to a lesser degree, *Firmicutes* (6.1%), and *Actinobacteria* (3.5%), while 0.6%

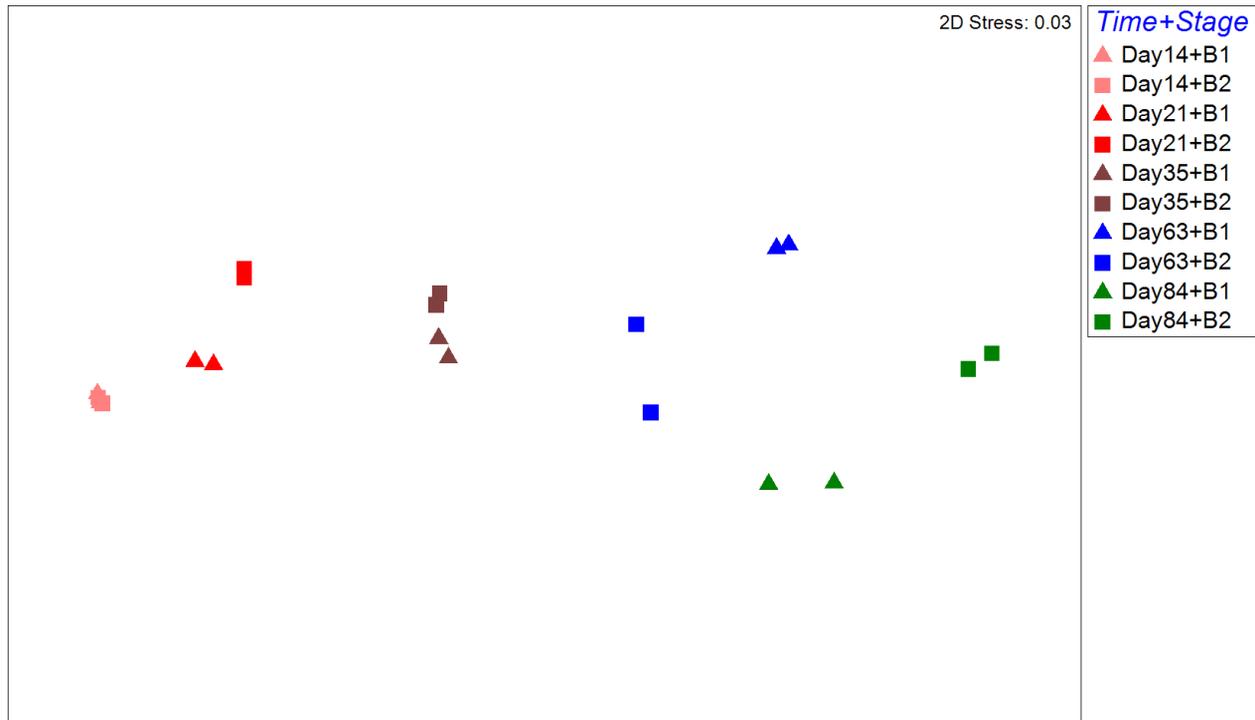
of the sequences were unclassified (Figure 21). Similar community compositions have been observed from a full-scale biofilter treating swine house exhaust air (Kristiansen *et al.* 2011b), and also from other air filter biofilms (Borin *et al.* 2006; Friedrich *et al.* 2002; Friedrich *et al.* 2003). These results indicate that the bacterial biofilm community of biofilter was specialized and adapted to the unique environmental conditions. A non-metric multidimensional scaling (NMDS) using Bray-Curtis distance showed that bacterial biofilm community on biofilter was strongly influenced by time (Figure 22). A permutational multivariate analysis of variance (PERMANOVA) results showed that bacterial biofilm community composition of biofilter was strongly influenced by time ( $F = 52.6$ ,  $P < 0.0001$ ; Table 10) which explained 84% of the variation in community composition. The filtration stage ( $F = 5.1$ ,  $P = 0.02$ ; Table 10) and the interaction between time and filtration stage ( $F = 6.2$ ,  $P = 0.0003$ ; Table 10) also significantly influenced the bacterial community composition, however, the explained proportion of the variations were lower compared to time (filtration stage = 2%, time x filtration stage = 11%). The successional change in bacterial biofilm community of biofilter with time was reported earlier (Portune *et al.* 2015), and it has been also observed that bacterial community structure changes along the filtration stages of the biofilter (Kristiansen *et al.* 2011b).



**Figure 20.** Relationship between time and biofilter reduction efficiency of various odorous compounds.



**Figure 21.** Relative abundances of dominant bacterial taxa across time point and filter stage.



**Figure 22.** NMDS plot of Bray–Curtis dissimilarities between samples at different time point and two stages

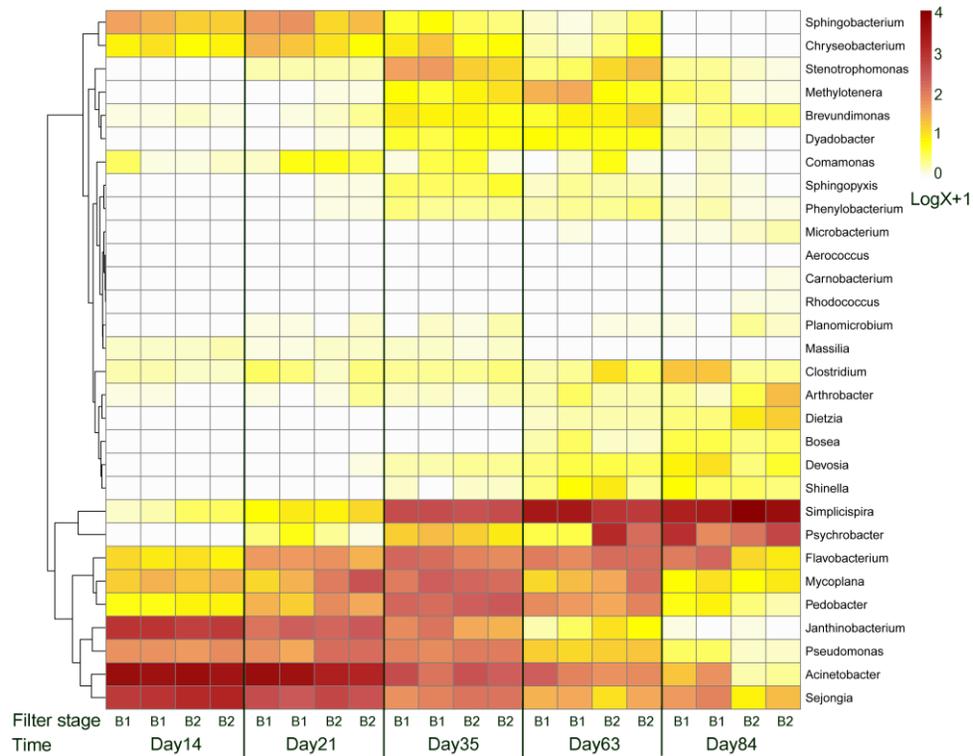
**Table 10.** Results of multivariate PERMANOVA verify significant differences in bacterial community composition between treatment groups.

	Bacterial community composition				
	d.f	MS	F. Model	R <sup>2</sup>	<i>P</i> value
Time	4	0.74	52.62	0.84	0.0001
Filter stage	1	0.07	5.07	0.02	0.02
Time x Filter stage	4	0.09	6.23	0.11	0.0003
Residuals	10	0.01		0.04	
Total	19			1.00	

During the initial time point of the experiment (day 14) *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria* and *Bacteroidetes* were the most abundant bacterial phyla across both primary and secondary stage filters (Figure 21). At the mid time point of the experiment (day 35) the relative abundance of *Gammaproteobacteria* decreased with increase in the relative abundance of *Alphaproteobacteria* and *Betaproteobacteria*, however, the relative abundance of *Bacteroidetes* remained similar (Figure 21). At the late time point (day 84), the relative abundance of *Betaproteobacteria* increased further compared to mid time point and the relative abundance of *Gammaproteobacteria* and *Bacteroidetes* were decreased, however, the relative abundance of *Alphaproteobacteria* did not show much variation at late time point compared to mid time point (Figure 21). The members of the *Proteobacteria* and *Bacteroidetes* are reported to degrade various odorants (Ralebitso-Senior *et al.* 2012), and dominance of different subgroups of the bacteria phyla at the later time of the experiment made biofilter more efficient in reduction of a wide variety of odorants. Interestingly, the relative abundance of the phylum *Actinobacteria* increased sharply at late time point of the experiment (Figure 21). The members of *Actinobacteria* are known to degrade butyric acid and dimethyl disulfide (Kristiansen *et al.* 2011a).

The comparison of relative abundance of bacterial biofilm community at the genus level also revealed many apparent relationships to time (Figure 23). The heat map of 30 most abundant bacterial genera showed that among the dominant genera in these samples, no single genus was abundant at all time point, although each shows its own

pattern peaking at early, mid or late time points (Figure 23). Most of the abundant genera found in this study are known to degrade various odorants (Table 11).



**Figure 23.** Heat map showing the relative abundance ( $\log x + 1$  transformed) of 30 most dominant bacterial genera at each time point and filter stage (B1=Primary stage and B2=Secondary stage).

**Table 11.** The known odorant degrading bacterial genera found in this study.

<b>Genera</b>	<b>Odorant/class</b>	<b>References</b>
<i>Acinetobacter</i>	Hydrogen sulfide	(Omri <i>et al.</i> 2011)
<i>Arthrobacter</i>	Hydrogen sulfide, ammonia, methylamine, dimethylamine and trimethylamine	(Chung <i>et al.</i> 2001; Ho <i>et al.</i> 2008)
<i>Clostridium</i>	Nitric oxide	(Chen <i>et al.</i> 2009)
<i>Devosia</i>	VOC	(Friedrich <i>et al.</i> 2002)
<i>Dietzia</i>	Dimethyl sulfide and butyric acid	(Kristiansen <i>et al.</i> 2011a)
<i>Flavobacterium</i>	Butyric acid, dimethyl disulfide and other VOC	(Friedrich <i>et al.</i> 2002; Kristiansen <i>et al.</i> 2011a)
<i>Microbacterium</i>	Dimethyl sulfide, butyric acid and dimethyl disulfide	(Kristiansen <i>et al.</i> 2011a; Shu & Chen 2009)
<i>Pedobacter</i>	VOC	(Friedrich <i>et al.</i> 2002)
<i>Pseudomonas</i>	Hydrogen sulfide, dimethyl sulfide, ammonia and VOC	(Omri <i>et al.</i> 2011; Shu & Chen 2009)
<i>Psychrobacter</i>	Ammonia, hydrogen sulfide, dimethylamine, trimethylamine, isobutyric acid	(Gutarowska <i>et al.</i> 2014)
<i>Rhodococcus</i>	Dimethyl sulfide, butyric acid, ethyl acetate, o-xylene, and VOC	(Aldric & Thonart 2008; Jeong <i>et al.</i> 2008; Kristiansen <i>et al.</i> 2011a)
<i>Simplicispira</i>	Dimethylamine	(Liao <i>et al.</i> 2015)
<i>Sphingobacterium</i>	Butyric acid, dimethyl disulfide, dimethylamine and VOC	(Friedrich <i>et al.</i> 2002; Kristiansen <i>et al.</i> 2011a; Liao <i>et al.</i> 2015)
<i>Stenotrophomonas</i>	Ammonia and VOC	(Friedrich <i>et al.</i> 2002)

#### **4.1.4 Conclusions**

In conclusion, biofilter system used in this study was found to reduce various odorants emission efficiently from swine farm facility. Bacterial biofilm community structure of biofilter exhibited a strong time successional pattern, and to a lesser extent, filtration stage and the interaction between time and filtration stage also lead to a significant variation in community structure of bacterial biofilm. Certain bacterial phyla and genera with ability to degrade various odorants got enriched at later time point of the experiment which might result in reduction of a wide variety of odorants. Analysis of the bacterial biofilm community of biofilter system in the present study represents an important first step toward understanding the stability and efficiency of such biofilters.

## GENERAL CONCLUSIONS

The high animal densities in SCBs lead to poor indoor air quality. The airborne contaminants present in SCBs affect both animal and human health. Although, the indoor airborne contaminants have been analyzed in several studies in SCBs, relatively little is known about the factors influencing the abundance and community composition of bioaerosols in SCBs. Also, despite growing awareness of health risk in the neighboring resident community, the mitigation strategies of airborne contaminants emission from SCBs are poorly studied. This study investigated the indoor bioaerosols community structure and diversity in SCBs, and how the bioaerosol communities are affected by manure removal system and seasonal variations. In this study, a biofiltration system was also used to study how and to which extent it helps in mitigation of airborne contaminants emissions from SCBs.

At first, the effect of three different types of manure removal systems (deep-pit manure removal with slats, scraper removal system, and deep-litter bed system) was investigated on the abundance and composition of airborne biotic contaminants of SCBs. The manure removal system was found to strongly influence the abundance and community composition of airborne biotic contaminants of SCBs. The airborne biotic contaminants were most abundant in SCBs equipped with a deep pit with slats. *Firmicutes* and *Actinomycetes* appear to be the dominant bacterial phyla in SCBs, and a comparison of relative abundances of these phyla together with dominant genera strongly supports the concept that individual bacterial lineages found in SCBs

are enriched to specific manure removal systems. The present study represents a first glimpse of the airborne biotic contaminants of SCBs with different types of manure removal systems using next-generation sequencing methods.

The next objective of this study was to investigate the seasonal variations in community composition and the diversity of airborne contaminants (bacteria, fungi and tetracycline resistance genes) in SCBs. The results indicate that seasons have an influence on the biotic contaminants abundance, community composition and diversity, in indoor air of SCBs. Seasonality was significantly associated with microclimate variables, indicating that indoor environmental conditions play an important role in structuring airborne biotic contaminants in SCBs. Several human allergen/pathogen related fungal genera were also detected in SCBs in both seasons, and in total, their relative abundance was higher in winter. These potential human allergen/pathogen related fungal genera present in the indoor air of SCBs may impact the health of farm workers, which suggests that better management practices are needed to minimize the risk of potential occupational health hazards in farm workers. However, it is to be noted that fungi are generally opportunistic pathogens, and it is very unlikely that these opportunistic fungal pathogens can infect healthy individuals.

In the final objective of this study, a biological air filter system was used to reduce the emissions of airborne contaminants from SCBs, and also investigated the successional development of bacterial biofilm community in the packing material of biofilter by using the Illumina Miseq sequencing platform. In this study, it has been observed that the

odorant reduction efficiency of biofilter was increased linearly with time. The results also indicate that bacterial biofilm community structure of biofilter exhibited a strong time successional pattern, and to a lesser extent, filtration stage and the interaction between time and filtration stage also lead to a significant variation in community structure of bacterial biofilm. Certain bacterial phyla and genera with ability to degrade various odorants got enriched at later time point of the experiment which might result in reduction of a wide variety of odorants.

Overall, it appears that patterns of community composition and diversity of bioaerosols in SCBs were strongly influenced by manure removal system and seasonal variations. It has been also observed in this study that biofilter could be used as efficient and cost effective option to reduce the emissions of airborne contaminants from SCBs. Together these results provide a baseline framework with which better management practices and regulations can be designed to minimize the potential health impact on both the farm workers and the public residing in close proximity to these buildings.

### **Significance of this study for swine farmers**

The following are the significance of this study for swine farmers:

- 1) In this study, it has been found that the manure removal system strongly influence the airborne biotic contaminants present in swine confinement buildings. Adoption of better management practices, such as proper cleaning of swine manure and optimizing the hosing conditions could minimize these airborne contaminants in SCBs.

- 2) Seasonal trend in airborne biotic contaminants is also recognized in this study, with winter season peaked in the abundance and diversity of airborne contaminants due to minimal ventilation rate to prevent the heat loss during winter. The abundance, composition, and diversity of these contaminants were found significantly associated with microclimatic parameters of SCBs, particularly air speed, PM<sub>2.5</sub> and TSP, which suggests that by controlling the microclimate parameters the concentrations of airborne biotic contaminants can be reduced to minimize potential health impacts on both livestock and humans working in SCBs.
  
- 3) Odorous gas emissions from SCBs have been of increasing concern in the South Korea. In this study, a biofilter system has been successfully used to reduce odorous gas emissions from an experimental swine farm facility. This biofilter system enriched the odor degrading native airborne microbial community of swine house. This biofilter system could be used as an efficient and cost effective option to reduce the emissions of odorous gases from SCBs.

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## APPENDIX



**Figure A1.** A Gilian air sampler (Sensidyne Inc., Clearwater, FL, USA) used in this study for collecting aerosol samples from SCBs.



**Figure A2.** (a) Outdoor and (b) indoor view of swine farm facility of Seoul National University at Suwon, South Korea.



**Figure A3.** A two-stage biofilter installed next to an experimental swine farm facility of Seoul National University at Suwon, South Korea.



**Figure A4.** Gas chromatograph-mass spectrometer (Agilent GC6890N/5975C MS, Youngin, Korea) used in this study to determine the concentration of odorous gases.

## ABSTARCT IN KOREAN

무창돈사 (SCB) 내 부유세균의 풍부도 및 군집구성 그리고 그 감소에 영향을 미치는 주요 요인에 대한 연구는 거의 이루어진 적이 없다. 본 연구에서는 돈사 내에서 실내 부유세균 군집 구조와 다양성에 대한 연구를 차세대 염기서열 분석 기술을 이용하여 수행하였다. 이 차세대 염기서열 분석법을 이용하여 유형별 돈분뇨 제거시스템 및 계절적 변화에 따른 부유세균 군집에 대한 영향의 영향을 조사하였다. 또한 본 연구에서는 생물학적 여과시스템을 사용하여 이것이 돈사로부터 배출되는 공기오염 물질의 완화에 얼마나 도움이 되는지 및 그 방법에 대하여 연구하였다.

Cultivation-independent 방법을 이용한 분뇨제거 시스템 유형 (슬랫-피트 분뇨제거시스템, 스크레이퍼 분뇨제거시스템, 깔개시스템)이 돈사 내 부유생물 오염물질의 풍부도 및 조성에 미치는 영향을 연구하였다. 16S rRNA 및 유전자 여섯 테트라사이클린 내성 유전자 (tetB, tetH, tetZ, tetO, tetQ 및 tetW) 의 풍부도 존재비는 실시간 PCR 을 사용하여 정량화하였다. 16S rRNA 유전자 및 tetB 유전자를 제외한 테트라사이클린 내성 유전자의 풍부도는 슬랫-피트 돈사에서 유의하게 높았다. 이러한 결과는 기존의 배양 기반 연구의 결과와 대조된다. pairwise Bray-Curtis distances 방법으로 측정된 부유세균의 군집조성은 분뇨제거시스템 유형에 따라 유의한 변화를 보였다. 16S rRNA 기반 pyrosequencing 결과, *Firmicutes* (72.4%)가 우점군으로 *Lactobacillus* 와 함께 주요 종으로 나타났고, 반면 *Actinobacteria* 는 검출세균의 10.7 %로 나타났다. *Firmicutes* 는 칸막이가 있는 슬랫-피트 분뇨제거시스템 돈사 내에 더 많이 분포하였고, 반면 *Actinobacteria* 는 깔개돈사 내에 매우 풍부하게 분포하였다. 전반적으로, 이 연구의 결과는 무창돈사의 가축분뇨처리시스템 유형이 내 공기 중 부유세균 의 풍부도와 구성을 구조화하는데 중요한 역할을 함을 제시하였다.

무창돈사 내 부유세균의 계절적 역학에 대해서는 거의 알려져 있지 않다. 본 연구에서는 7개 농장의 돈사 내 부유세균을 여름과 겨울에 한 차례 씩 방문하여 관찰하였다. 16S rRNA 유전자, V3 영역에 대한 paired-end Illumina 염기서열 분석방법으로 세균 군집 구성과 다양성의 계절적 변화를 조사하였다. 16S rRNA의 유전자와 여섯 개의 테트라사이클린 내성 유전자(tetB, tetH, tetZ, tetO, tetQ 및 tetW)의 풍부도를 실시간 PCR을 이용하여 정량화하였다. 박테리아 풍부도, 군집구성 및 다양성은 겨울에 최대로 분석되어 강한 계절적 양상을 보였다. 무창돈사 내 미기상 변수 특히 유속, PM2.5 및 총부유 입자(TSP)가 세균의 풍부도 및 군집구성, 부유세균의 다양성에 유의한 상관관계가 있는 것으로 나타났다. 계절적 변화도 네 가지 테트라사이클린 내성 유전자, tetH, tetO, tetQ 및 tetW에서 관찰되었다. 이러한 내성 유전자의 발생빈도는 겨울 동안 수집된 표본에서 유의하게 높았으며, 또한 유속, PM2.5 및 TSP와 유의한 상관관계가 관찰되었다. ~~있었다.~~ 결론적으로, 이러한 결과는 무창돈사 내 부유세균 계절적 동향을 보이는 것으로 분석되며, 이들은 무창돈사 미기상 변수들과 상관 있음을 알 수 있었다.

무창돈사 내에서 계절의 변화가 부유 곰팡이의 군집 조성 및 다양성에 미치는 영향에 대해서도 연구하였다. 부유물 표본은 겨울 및 여름에 일곱 개의 양돈장에서 수집하였다. 리보솜 유전자의 내의 ITS region 1은 paired-end Illumina 염기서열 분석방법으로 서열화하였다. 세균과 마찬가지로, 실내 부유곰팡이의 군집구성 및 다양성은 계절변화에 의해 영향을 받음을 관찰하였다. 그러나, 알파와 베타 다양성은 서로 매우 다른 양태를 보였는데, 알파 다양성은 겨울에 정점을, 베타 다양성은 여름에 정점을 나타내었다. 여러 인체 알레르기 항원/병원체 관련 곰팡이 종(種)이 무창돈사 내에서 확인되었다. 이러한 인체 알레르기 항원/병원체 관련 곰팡이 종 중, *Candida*, *Aspergillus*, *Pichia*, *Trichosporon*은 계절에 따라 크게 변화하였다. 일반적으로, 인체 알레르기

항원/병원체 관련 곰팡이 종의 상대빈도는 여름보다 겨울에 더 높았다.

무창돈사로부터 배기(排氣)의 오염물질을 희석시키는 데는 바이오필터시스템이 경제적인 설비로 알려져 있다. 그러나, 공기흐름 내 존재하는 오염 물질의 분해에 도움이 되는 바이오 필터 표면에 고정된 세균 바이오 필름내의 군집구조에 대해서는 알려져 있지 않다. 생물학적 공기 여과시스템은 양돈사 내 공기 중 오염물질의 배출을 감소시키기 위해 사용되며, 또한 Illumina Miseq 염기 서열 분석기술을 이용하여 바이오 필터의 포장재 내 세균 바이오 필터의 군집의 발달과정에 대하여 조사하였다. 그 결과 바이오 필터의 악취감소 (냄새제거) 효율이 시간에 따라 선형적으로 증가함을 관찰하였다. 연구결과에 따르면 바이오 필터의 박테리아 군집에 존재하는 바이오 필터 구조 또한 강한 시간 연속적인 양상을 보이고, 보다 적게는, 여과 단계와 시간 및 여과 단계 사이의 상호작용 또한 세균 바이오필터의 군집 내에서 매우 다양하게 나타났다. 각종 악취물질을 분해하는 능력을 지닌 특정 세균 문(門) 및 종(種)이 실험 후기 시점에 농축되어 다양한 종류의 악취가 감소됨을 관찰하였다.

결론적으로, 실내 부유세균의 군집 구성 및 다양성은 분뇨제거 시스템 및 계절변동에 따라 크게 달라지는 것으로 밝혀졌다. 또한 바이오필터시스템이 효율적으로 무창돈사에서 발산되는 다량의 악취기체의 배출을 감소시키는 것이 관찰되었고, 세균 바이오필름의 군집의 천이 및 악취 제거의 상관관계는 축사내 작업자와 축사주위 정주민에 대한 잠재적 건강위해적 영향을 최소화하기 위한 더 나은 관리 전략 수립에 도움이 될 수 있을 것이다.

**키워드:** 부유세균, 바이오필터, 세균, 곰팡이, 일루미나, ITS, 가축분뇨처리시스템, pyrosequencing, 계절변화, 무창돈사, 16S rRNA 유전자.

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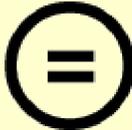
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Doctoral Thesis

**Assessment and Mitigation of  
Airborne Contaminants from Swine  
Confinement Buildings**

February 2016

Graduate School of Seoul National University

Department of Agricultural Biotechnology

Priyanka Kumari

**Assessment and Mitigation of  
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by

**Priyanka Kumari**

under the supervision of

**Professor Hong Lim Choi, Ph.D.**

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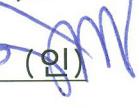
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# **ABSTRACT**

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The dominant factors influencing the abundance and community composition of bioaerosols in swine confinement buildings (SCBs) and their mitigation have been poorly studied. In this study, the indoor bioaerosols community structure and diversity were investigated in SCBs by using next generation sequencing platforms (454-pyrosequencing and Illumina). The effects of manure removal system and seasonal variations were investigated on bioaerosol communities obtained through NGS platforms. In this study, a biofiltration system was also used to explore how and to which extent it helped in mitigation of airborne contaminants emissions from SCBs.

The effect of manure removal systems (deep-pit manure removal with slats, scraper removal system, and deep-litter bed system) was studied on abundance and composition of airborne biotic contaminants in SCBs using cultivation-independent methods. The abundances of 16S rRNA genes and six tetracycline resistance genes (tetB, tetH, tetZ, tetO, tetQ, and tetW) were quantified using real-time PCR. The abundance of 16S rRNA gene and tetracycline resistance genes were significantly higher in SCBs equipped with a deep-pit manure removal system with slats, except for tetB gene. This observation contrasts with the trend found previously by culture-based

studies. The aerial bacterial community composition, as measured by pairwise Bray–Curtis distances, varied significantly according to the manure removal system. 16S rRNA-based pyrosequencing revealed *Firmicutes* (72.4 %) as the dominant group with *Lactobacillus* as the major genus, while *Actinobacteria* constituted 10.7 % of the detectable bacteria. *Firmicutes* were more abundant in SCBs with deep-pit with slats, whereas *Actinobacteria* were highly abundant in SCBs with a deep-litter bed system. Overall, the results of this study suggested that the manure removal system played a key role in structuring the abundance and composition of airborne biotic contaminants in SCBs.

Little is known about the seasonal dynamics of biotic contaminants in SCBs. The biotic contaminants of seven SCBs were monitored during one visit in winter and one during summer. Paired-end Illumina sequencing of the 16S rRNA gene, V3 region, was used to examine seasonal shifts in bacterial community composition and diversity. The abundances of 16S rRNA genes and six tetracycline resistance genes (*tetB*, *tetH*, *tetZ*, *tetO*, *tetQ*, and *tetW*) were also quantified using real-time PCR. Bacterial abundances, community composition and diversity showed strong seasonal patterns defined by winter peaks in abundance and diversity. Microclimatic variables of SCBs, particularly air speed, PM<sub>2.5</sub> and total suspended particles (TSP) were found significantly correlated to abundances, community composition, and diversity of bacterial bioaerosols. Seasonal fluctuations were also observed for four tetracycline resistance genes, *tetH*, *tetO*, *tetQ*, and *tetW*. The frequency of occurrences of these resistance genes were significantly higher in samples collected during winter and was also significantly correlated with air speed, PM<sub>2.5</sub> and

TSP. Overall, the results indicate that biotic contaminants in SCBs exhibit seasonal trends, and these could be associated with the microclimatic variables of SCBs.

Seasonal variations in community composition and diversity of airborne fungi were also studied in SCBs. Aerosol samples were collected from seven commercial swine farms in winter and summer. The internal transcribed spacer region 1 (ITS 1) of the ribosomal genes was sequenced using paired-end Illumina sequencing. Similarly to bacteria, indoor airborne fungal community composition and diversity were influenced by seasonal variations. However, the alpha and beta diversities showed very different patterns from one another, whereby alpha diversity peaked in winter and beta diversity peaked in summer. Several human allergen/pathogen related fungal genera were also identified in SCBs. Among these human allergen/pathogen related fungal genera, *Candida*, *Aspergillus*, *Pichia*, and *Trichosporon* varied significantly between seasons. In general, the relative abundance of human allergen/pathogen related fungal genera was higher in winter than in summer.

Biofiltration is known as one of cost-effective technology for treating the ventilation exhaust air from livestock buildings. However, little is known about the bacterial biofilm community immobilized in the packing material of biofilter which helps in breaking down of the contaminants present in air stream. A biological air filter system was used to reduce the emissions of airborne contaminants from SCBs, and also investigated the successional development of bacterial biofilm community in the packing material of biofilter by using the Illumina Miseq sequencing platform. It has been observed that the odorant

reduction efficiency of biofilter was increased linearly with time. The results also indicated that bacterial biofilm community structure of biofilter exhibited a strong time successional pattern, and to a lesser extent, filtration stage and the interaction between time and filtration stage also led to a significant variation in community structure of bacterial biofilm. Certain bacterial phyla and genera with ability to degrade various odorants got enriched at later time point of the experiment which might result in reduction of a wide variety of odorants.

Overall, it has been found that the indoor bioaerosol community composition and diversity are to a large extent structured by manure removal system and seasonal variations. It has been also observed that biofilter system efficiently reduced the emissions of a large number of odorous gases from SCBs, and the correlations established between bacterial biofilm community succession and odorants removal could be helpful in establishing better management strategies to minimize the potential health impacts on both the farm workers and the public residing in close proximity to these buildings.

**Keywords:** bioaerosols, biofilter, bacteria, fungi, Illumina, internal transcribed spacer, manure removal system, pyrosequencing, seasonal variations, swine confinement buildings, 16S rRNA gene.

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## **ABBREVIATIONS**

OTU: Operational taxonomic unit

PCR: Polymerase chain reaction

NGS: Next generation sequencing

SRA: Short read archive

rRNA: Ribosomal ribonucleic acid

NMDS: Non-metric multidimensional scaling

BLAST: Basic local alignment search tool

RDP: Ribosomal database project

DNA: Deoxyribonucleic acid

PERMANOVA: Permutational multivariate analysis of variance

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# **CHAPTER 1. Airborne Contaminants Present in Swine Confinement Buildings and Their Control: An Introduction**

## **1.2. Airborne contaminants present in swine confinement buildings**

Swine farming is revolutionized by the use of confined buildings all over the world. However, high animal densities in these confined environments lead to poor indoor air quality. The decomposition of animal feces and urine generates various gases (O'Neill & Phillips 1992), while feed and bedding materials, skin debris and dried manure produce particulate matter (PM) which helps in transportation of adsorbed microorganisms and endotoxins (Cabra-López *et al.* 2010). These airborne contaminants present in swine confinement buildings (SCBs) affects both animal and human health. Furthermore, the indoor air of SCBs is regularly ventilated to the outdoor environment, and the airborne contaminants present in the ventilated air can cause detrimental effects to the public residing in close proximity to these buildings. The airborne contaminants present in the SCBs are broadly categorized into three different types: particulate matter, gaseous compounds and bioaerosols. Particulate matter and gaseous compounds are discussed in following subsections, whereas bioaerosols are discussed in a separate section as most of the work in this study is on bioaerosols.

### **1.2.1. Particulate matter**

Particulate matter is a mixture of various types of pollutants with different physical, chemical and biological characteristics, and these characteristics determine the environmental and health impact of particulate matter (EPA 2004). The concentrations of PM are generally

10-100 times greater in livestock buildings compared to other indoor environments and PM carries various gases and microorganisms (Zhang 2004). Most of the PM present in SCBs are generated from feed materials (Donham *et al.* 1986; Heber *et al.* 1988; Honey & McQuitty 1979; Takai *et al.* 1998). The other possible sources of PM are skin debris and fecal materials of animals (Heber *et al.* 1988; Honey & McQuitty 1979). The concentrations of PM are also linked to swine activity (Kim *et al.* 2008b); with nursery swine houses have higher PM concentrations than finishing houses (Attwood *et al.* 1987). The main proportion of respirable PM has a diameter less than 5  $\mu\text{m}$  (Gustafsson 1999), and based on the site of deposition in body there are following types of PM (Carpenter 1986): PM > 10  $\mu\text{m}$ : nasal passage; PM 5-10  $\mu\text{m}$ : upper respiratory tract; PM < 5  $\mu\text{m}$ : lungs. It has been studied earlier in several studies that the respirable fraction of PM in SCBs varies from 7% to 13% (Donham 1986b; Gustafsson 1999). The presence of PM in SCBs can lead to severe respiratory diseases and loss of respiratory capacities in farm workers especially in young farm workers (Zejda *et al.* 1993). The exposure time to the indoor environment of SCBs can be associated to changes in respiratory capacities (Donham *et al.* 1989).

### **1.2.1. Gaseous compounds**

The most abundant gaseous compounds in the air of SCBs are ammonia ( $\text{NH}_3$ ), carbon dioxide ( $\text{CO}_2$ ), methane ( $\text{CH}_4$ ), hydrogen sulfide ( $\text{H}_2\text{S}$ ) and various volatile organic compounds (VOCs). The representative VOCs present in SCBs are sulfuric compounds, volatile fatty acids (VFAs), indolics and phenolics (Cai *et al.* 2006; Kai &

Schäfer 2004). Approximately 331 different VOCs and gaseous compounds have been reported in SCBs (Schiffman *et al.* 2001). These gaseous compounds are generated mainly due to microbial activity. In SCBs, the hydrolysis of urea led to generation of NH<sub>3</sub> from urine and swine slurry and this reaction is catalyzed by the enzyme urease (Mobley & Hausinger 1989). The level of dietary crude proteins influences NH<sub>3</sub> emissions as these are the primary source of nitrogen (Le *et al.* 2009), and it has been shown that NH<sub>3</sub> emissions can be limited by the reduction in dietary crude protein (Portejoie *et al.* 2004). Many other gaseous compounds (CH<sub>4</sub>, CO<sub>2</sub>, and H<sub>2</sub>S) are also produced by the microbial action on swine manure stored in manure pits under the building (Donham 1986a; Pedersen *et al.* 2008). Due to intensive animal stocking in confinement buildings, some gaseous compounds (NH<sub>3</sub> and H<sub>2</sub>S) can accumulate rapidly and become a respiratory hazard for both farm workers and animals. It has been also reported that the emission of the gaseous compounds to the outdoor environment are associated with mental health consequences, such as increased tension, depression, fatigue, confusion, and mood changes in members of surrounding communities (Schiffman *et al.* 1995; Schiffman & Williams 2005).

### **1.3. Bioaerosols and their characterization**

Bioaerosols comprises the biological particulates such as bacteria, fungi, viruses, endotoxin, and mycotoxin etc. suspended in air (Cox & Wathes 1995). Depending on the source, aerosolization mechanisms, and environmental conditions, bioaerosols can vary in

size (20 nm to >100 µm) and structure (Pillai & Ricke 2002). The inhalable fraction (1–10 µm) of bioaerosol is of primary concern because it can reach to deeper part of the respiratory tract (Stetzenbach *et al.* 2004). Both liquid and dry materials can act as source for bioaerosols. The usage of antibiotics in swine farms has promoted the development and abundance of antibiotic resistance in microbes (Blake *et al.* 2003; Zhu *et al.* 2013), which can become aerosolized within the SCBs. Antibiotic resistant genes (ARGs) can be transferred to pathogens through transformation or phage-mediated transduction, and/or by conjugation, posing a serious threat to public health. Pathogenic bioaerosols present in confinement buildings can cause direct harm to both farmworkers and animals. Bacterial pathogenic bioaerosols in SCBs can cause infectious and allergic diseases such as pneumonia, asthma, and rhinitis in workers and pigs (Donham *et al.* 1990; Pearson & Sharples 1995). The known allergenic, toxic, and inflammatory responses are caused by exposure not only to the viable but also to the non-viable microorganisms present in the air (Robbins *et al.* 2000). The presence of some pathogenic bioaerosols has been detected over long distances from their emission site which were found capable to infect healthy animals intramuscularly or intratracheally (Otake *et al.* 2010). It is clear that the swine confinement buildings are significant point sources of outdoor air contamination that can potentially pose a serious problem to public health.

### **1.2.1. Detection of bioaerosols by using cultivation-dependent approach**

Cultivation-dependent approach has been used initially to characterize the bioaerosols associated with SCBs. The predominant organisms cultured from bioaerosols of SCBs were bacteria and majority of the bacterial isolates from SCBs were Gram-positive species (Attwood *et al.* 1987; Chang *et al.* 2001; Crook *et al.* 1991; Donham *et al.* 1986; Mackiewicz 1998). The dominance of Gram-positive species in bioaerosols of SCBs suggested that swine feces are the primary source of the bacteria in the bioaerosols of SCBs, because 90% of the bacteria isolated from the feces of adult swine are reported as gram positive (Salanitro *et al.* 1977). The assessment of airborne bacteria in SCBs across the United States, Canada, The Netherlands, Sweden, Poland, and the United Kingdom showed that their concentration varied from  $10^5$  to  $10^7$  CFU/m<sup>3</sup> (Attwood *et al.* 1987; Clark *et al.* 1983; Cormier *et al.* 1990; Donham *et al.* 1989; Donham *et al.* 1986; Mackiewicz 1998; Nehme *et al.* 2008). It has been also found that the level of airborne bacterial count in SCBs showed seasonal variation with their concentrations peaking in summer season (Nehme *et al.* 2008).

The predominant bacterial genera identified in SCBs were *Aerococcus*, *Bacillus*, *Enterococcus*, *Micrococcus*, *Moraxella*, *Pseudomonas*, *Staphylococcus*, *Streptococcus* (Cormier *et al.* 1990; Crook *et al.* 1991; Predicala *et al.* 2002). The fungal colony forming units (cfu) reported in SCBs varied from  $10^3$  cfu/m<sup>3</sup> to  $10^6$  cfu/m<sup>3</sup> (Chang *et al.* 2001; Clark *et al.* 1983; Predicala *et al.* 2002). *Aspergillus*, *Cladosporium* and *Penicillium* were the most abundant fungal genera in SCBs. Several other fungal genera have been also detected in SCBs such as *Alternaria*, *Fusarium*, *Verticillium*, and

*Geotrichum*. Manure removal system in SCBs was shown to influence the indoor air fungal concentrations and emissions (Kim *et al.* 2008c). Keratinophilic (*Scopulariopsis brevicaulis*) and toxigenic fungi (*Aspergillus*, *Fusarium*, and *Penicillium* genera and *Stachybotrys chartarum*) were also detected on SCBs, suggesting a potential occupational health threat (Viegas *et al.* 2013). Jo *et al.* (2005) studied airborne fungi concentrations in swine sheds and reported that the summer concentrations of total fungi and fungal genera inside the swine sheds were substantially higher than the winter values. The initial characterization of bioaerosols based on cultivation-dependent techniques provided several important information about bioaerosols, however, cultivation-dependent techniques contain an inherent bias, as only the viable microbes that can be grown in culture are characterized. Furthermore, the majority of microorganisms cannot be cultured using standard cultivation techniques (DeLong & Pace 2001; Torsvik *et al.* 1996). This bias in cultivation led to overestimation of microorganisms that are easily cultured through standard cultivation techniques.

### **1.2.2. Detection of bioaerosols by using cultivation-independent metagenomic approach**

Cultivation-independent metagenomic approach bypasses the need of cultivation of microorganisms. Cultivation-independent metagenomic approach is mainly grouped into two categories; shotgun metagenomics and sequence-driven metagenomics based on their random and targeted sequencing strategies, respectively. There have been only few studies that have investigated the bioaerosol samples

collected from SCBs using cultivation-independent metagenomic approach. Nehme et al. (2009) examined bacterial and archaeal biodiversity using cultivation-independent metagenomic approach. Nehme et al. (2009; 2008) showed that the total bacterial concentrations in bioaerosol samples were 1,000 times higher than the concentration of airborne culturable bacteria. They also found that bacterial and archaeal concentrations in bioaerosol samples were significantly lower in summer than values obtained in winter, and bioaerosol microbial populations bear close resemblance to the fecal microbiota of confined pigs (Nehmé *et al.* 2009; Nehme *et al.* 2008). In another study, Kristiansen et al. (2012) also applied cultivation independent metagenomic approach to estimate the diversity and abundance of bioaerosolic bacteria and fungi in SCBs. They found more diverse bacterial and fungal community in bioaerosols of SCBs, and suggested that this could potentially create poor indoor air quality in SCBs. However, these used denaturing gradient gel electrophoresis and 16S-cloning-and-sequencing approach to characterize the microbial community in bioaerosol samples, which lack resolution and throughput, respectively, compared to next generation sequencing based methods (Bartram *et al.* 2011; Bent *et al.* 2007; MacLean *et al.* 2009).

The recent development of next-generation sequencing (NGS) technologies has revolutionized the cultivation-independent metagenomic approach, enabled researchers to obtain much more DNA information from highly complex microbial communities (Mardis 2008). These NGS platforms such as 454 pyrosequencing, Illumina, Solid<sup>TM</sup> systems, and Ion Torrent<sup>TM</sup> are much faster and cheaper than

the traditional Sanger method in DNA sequencing. In a recent study, Hong et al. (2012) used 454-pyrosequencing to analyze the airborne biotic contaminants in pig and poultry confinement buildings sampled from different climate conditions and found that the different livestock as well as production phase were associated with distinct airborne bacterial communities. In an another recent study, Boissy et al. (2014) used shotgun pyrosequencing metagenomic analyses of DNA from settled surface dusts from swine confinement facilities and grain elevators and found that the domain “Bacteria” predominates in bioaerosols followed by the domain “Eukaryota” and “Archaea”. The NGS based cultivation-independent metagenomic approach could next be applied to understand and determine how manure removal system and seasonal variations affect the bioaerosols in SCBs, which might ultimately be helpful in establishing better management strategies to minimize the potential health impacts on both livestock and humans working in this environment.

### **1.3. Airborne contaminants mitigation strategies**

The mitigation strategies of airborne contaminants from SCBs are classified mainly into two groups: prevention of pollutant formation and abatement of pollutants.

#### **1.3.1. Particulate matter mitigation**

The emissions of PM from SCBs can be reduced by preventing their formation (Martin *et al.* 1996). The use of feed additives, oil or water spraying, adequate ventilation and regular management of

manure all can minimize the PM emissions from SCBs (Maghirang *et al.* 1995). The use feed additives such as animal fat or vegetable oil has been shown to reduce both inhalable and respirable fractions of PM (Guarino *et al.* 2007). Peason and Sharples (1995) suggested that altering the shape of the feed, composition and its delivery system can reduce the PM generation from feed materials. The spraying of oil and water mixtures together with a feed enhanced by fat have been also shown to reduce the PM concentrations (Pedersen *et al.* 2000; Takai & Pedersen 2000). The use of filtration system has shown not only to remove PM efficiently (up to 99% efficiency), but also appears to be the cost effective option (Owen 1982a, b).

### **1.3.2. Gaseous compound mitigation**

The gaseous compounds in SCBs are mainly originated from feces and urine therefore the production of gaseous compounds can be reduced by modifying feed. It has been shown that for each percent reduction of crude protein content in feed can reduce NH<sub>3</sub> emissions by 9.5% (Le *et al.* 2009). The use of manure additive can also reduce the emission of gaseous compounds. Additions of nitrites and molybdates to manure have shown to reduce H<sub>2</sub>S emissions by inhibiting sulfate reducing bacteria and oxidizing sulfide (Predicala *et al.* 2008). Also, use of peroxides is shown to reduce the emissions of gaseous compounds derived from phenolics (Govere *et al.* 2005). Regular management of manure in pits under house floor has shown to reduce the emissions of NH<sub>3</sub> and other gaseous compounds (Guingand 2000). The use of essential oils has shown to reduce the intensity of odorous gaseous compounds (Kim *et al.* 2008a). Biofiltration system is also

used to reduce the emissions of gaseous compounds from SCBs, and it has been shown that biofilter allows a 64–69% decrease in  $\text{NH}_3$  and an 85–92.5% reduction in other odorous gaseous compounds (Sheridan *et al.* 2002). The removal efficiency of biofilter has been shown to fluctuate with seasonal variation, however still biofiltration is the most cost-effective technology for treating the ventilation exhaust air (Nicolai & Janni 1997).

### **1.3.3. Bioaerosol mitigation**

The approaches mentioned earlier for PM and gaseous compound mitigations such as the use of feed additives, oil or water spraying, adequate ventilation and the use of biofiltration system can also be used to reduce and control the emissions of bioaerosols from SCBs. Gore *et al.* (1986) reported a 27% reduction in concentrations of airborne bacteria, when 5% soybean oil was added to the feed. Kim *et al.* (2006) sprayed several biological additives in pig houses and found that soybean oil spray significantly reduced total dust, airborne bacteria and fungi until 24h in SCBs. Air scrubbers and biofilters have been also reported to reduce the emissions of bioaerosols from SCBs. Aarnink *et al.* (2011) reported a 70% reduction in concentrations of total bacteria by using a sulfuric acid scrubber. Although, biofilters have been shown very efficient and cost effective in reducing odorous gaseous compounds, these are not consistent in reducing microorganisms (Seedorf & Hartung 1999). This could be probably due to the emission of microorganisms to the ambient air which colonized on the surface of biofilter.

## 1.4. Objectives of this study

While much has been done to monitor the indoor airborne contaminants in SCBs (Hong *et al.* 2012; Nehmé *et al.* 2009; Nehme *et al.* 2008), relatively little is known about the factors influencing the abundance and community composition of bioaerosols in SCBs. Also, despite growing awareness of health risk in the neighboring resident community, the mitigation strategies of airborne contaminants emission from SCBs are poorly studied. Therefore, there is a need to thoroughly characterize the airborne contaminants present in SCBs, and to develop a viable mitigation strategy to reduce their emission levels from SCBs.

The present study provides a thorough research on characterization and mitigation of airborne contaminants present in SCBs. The aerial bioaerosols community structure and diversity were analyzed using NGS based cultivation-independent metagenomic approach, and a biofiltration system was used in this study to mitigate the emission of airborne contaminants from SCBs. This study essentially sets out to answer the following questions:

- 1) How does the abundance and composition of airborne biotic contaminants vary in bioaerosols of SCBs equipped with different types of manure removal system?
- 2) Are the abundance and composition of airborne biotic contaminants more similar in bioaerosol samples collected during similar season? If so, are differences in abundance and composition better explained by variation in microclimate variables?

3) How and to what extent the emissions of airborne biotic contaminants from SCBs influence by biofiltration system?

# **CHAPTER 2. Effect of Manure Removal System on Airborne Biotic Contaminants Present in Swine Confinement Buildings**

## **2.1. Manure removal system influences the abundance and composition of airborne biotic contaminants in swine confinement buildings**

### **2.1.1. Introduction**

In recent years, there has been an increase in the use of confined buildings for swine farming in all over the world. Because of the high animal densities in these confined environments waste excreted from the pigs and residual feed accumulate indoors leading to poor indoor air quality. As a result, animals and workers are exposed to large quantities of volatile odorous compounds and a variety of bioaerosols that may impact their health (Cole *et al.* 2000; Yao *et al.* 2011; Zejda *et al.* 1994).

Bacteria are one of the major constituent of bioaerosols within SCBs with a mean airborne concentration of around  $10^5$  cfu m<sup>-3</sup> (Chang *et al.* 2001; Nehme *et al.* 2008). Aerosol bacterial pathogens in SCBs can cause infectious and allergic diseases such as pneumonia, asthma, and rhinitis in workers and pigs (Donham *et al.* 1990; Olson & Bark 1996; Pearson & Sharples 1995; Whyte 1993). The known allergenic, toxic, and inflammatory responses are caused by exposure not only to the viable but also to the non-viable microorganisms present in the air (Robbins *et al.* 2000). Furthermore, the development and abundance of antibiotic resistance in microbes has been promoted due to antibiotics usage in swine farming (Blake *et al.* 2003; Zhu *et al.* 2013), which can become aerosolized within SCBs. Because the indoor air is regularly ventilated from SCBs to external environment, these airborne contaminants can pose a serious problem to public health. Tetracycline

is the most commonly used antibiotic in livestock production in Korea (KFDA 2006). Although the use tetracycline in animal feed is completely prohibited in Korea from July 2011, still the resistance of tetracycline is prevalent in aerosol samples of SCBs (Kumari & Choi 2014).

Previous studies estimating bacterial bioaerosol content and levels in SCBs have been carried out predominately by cultivation-dependent methods (Lee *et al.* 2006; Nehmé *et al.* 2009; Predicala *et al.* 2002; Yao *et al.* 2010). However, culture-based techniques contain an inherent bias, as only the viable microorganisms that can be grown in the selected culture are identified. There have been only few studies that have investigated the bacterial bioaerosol community in SCBs using culture-independent methods. Nehme *et al.* (2009; 2008) examined bacterial and archaeal biodiversity using culture-independent methods and found that bioaerosol populations bear close resemblance to the fecal microbiota of confined pigs. In another study, Kristiansen *et al.* (2012) also applied cultivation-independent molecular approaches to estimate the diversity and abundance of bioaerosolic bacteria and fungi in SCBs and suggested that swine feces are the primary source of the bacteria in the bioaerosols. In recent studies using next-generation sequencing methods, it has been further validated that a major portion of the bacterial bioaerosols in SCBs is originated from excreted feces and soiled bedding material (Hong *et al.* 2012; Kumari & Choi 2014). Despite these revelations that most of airborne bacteria in SCBs are originated from swine feces, still relatively little is known about the effect of manure removal systems on abundance and composition of airborne biotic contaminants in SCBs.

Hence, this study essentially sets out to answer the following questions using the more accurate 16S rRNA based pyrosequencing and quantitative PCR methods:

(1) How does the abundance of airborne biotic contaminants vary in bioaerosols of SCBs equipped with the different types of manure removal system?

(2) What are the dominant bacterial taxa in bioaerosols of SCBs and how does the bacterial community composition vary in SCB bioaerosols equipped with the different types of manure removal systems?

## **2.1.2. Materials and Methods**

### **Characteristics of swine confinement buildings**

Samples were collected during the winter (January) season of 2013 from eight commercial swine farms in South Korea (Table 1). All sample collections took place in the growing/finishing rooms before the pigs were sent to the slaughterhouse. The ventilation methods employed in SCBs were mechanical ventilation (n=3) by exhaust fans on exit walls and natural ventilation (n=5) by means of a winch curtain. The number of animals housed in each sampling room ranged from 140 to 480, and the stocking density varied from 0.88 to 1.41 m<sup>2</sup>/ head (Table 1). The representative types of manure removal system equipped in these SCBs were deep-pit manure removal with slats (n=3), scraper removal system (n=3), and deep-litter bed system (n=2) (Figure 1). The deep pit manure system is composed of a deep manure pit under a fully or partially slatted floor. The manure is stored in the pit for a relatively long period of time before removal. The manure scraper removal system consists of a shallow manure pit with scrapers under a fully

slatted floor with the manure stored in shallow pit being removed several times a day from the SCBs. In the deep-litter bed system, pigs are kept on about 40-cm thick layer of a mixture of manure and litter such as sawdust, straw, or wood shavings. All pigs were fed with feeds from the Daehan Feed (Daehan Feed Co., Ltd., Korea) or from mills installed inside the farms.

### **Sample collection**

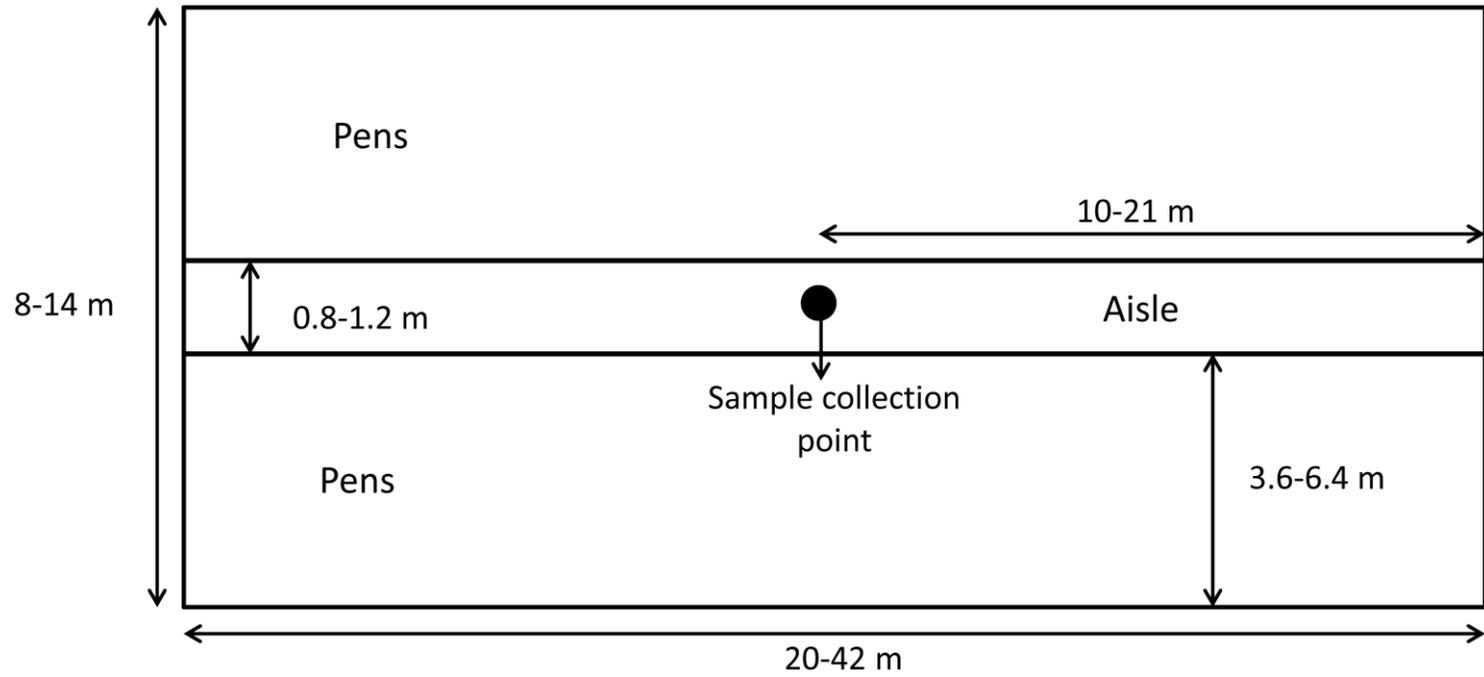
Aerosol samples were collected only once from the middle point in the aisle outside the pens at a height of 1.4 m above the floor (Figure 2). Air samples were captured on sterile 0.22- $\mu\text{m}$  cellulose nitrate filters (Fisher Scientific, Pittsburgh, PA) via a Gilian air sampler (Sensidyne Inc., Clearwater, FL, USA) (Figure A1) with a flow rate of 4  $\text{l min}^{-1}$  for 24 h. All components of the sampling system were sterilized in laboratory and aseptically assembled onsite prior sampling. All samples were immediately transported to the laboratory for subsequent molecular analyses. Blank filters were analyzed alongside sample filters to test for contamination, and following DNA extraction and amplification, blank filters were consistently found to be free of microbial contaminants.



**Figure 1.** Swine confinement buildings with (a) deep-pit manure removal with slats, (b) scraper removal system and (c) deep-litter bed system.

**Table 1.** Characteristics of the swine confinement buildings investigated in this study.

<b>Sample ID</b>	<b>Number of animals inside house</b>	<b>Stocking density m<sup>2</sup>/head</b>	<b>Manure removal system</b>	<b>Cleaning cycle</b>	<b>Ventilation mode</b>
F1	424	0.94	Deep-pit manure system with slats	About 6 months	Natural ventilation
F2	450	1.15	Deep-pit manure system with slats	About 6 months	Natural ventilation
F3	350	1.14	Deep-pit manure system with slats	About 6 months	Mechanical ventilation
F4	400	0.93	Manure removal by scraper	About 2 times in a day	Natural ventilation
F5	140	0.88	Manure removal by scraper	About 2 times in a day	Mechanical ventilation
F6	480	0.91	Manure removal by scraper	About 2 times in a day	Mechanical ventilation
F7	375	1.41	Deep-litter bed System	About 4 to 6 months	Natural ventilation
F8	352	1.13	Deep-litter bed system	About 4 to 6 months	Natural ventilation



**Figure 2.** Indoor plan view of aerosol collection point (black circle) in swine confinement buildings.

## **DNA extraction and quantitative PCR**

Bacterial DNA was extracted directly from the filters using the PowerSoil DNA isolation kit (MoBioLaboratories, Carlsbad, CA). Individual filters were aseptically cut into small pieces, loaded into the bead tube of the DNA extraction kit, and heated to 65 °C for 10 min followed by 2 min of vortexing. The remaining steps of the DNA extraction were performed according to the manufacturer's instructions. The purified DNA was resuspended in 50 µl of solution S6 (MoBio Laboratories) and stored at -20 °C until PCR amplification. The relative abundance of bacterial 16S rRNA genes and six tetracycline resistance genes (ribosomal protection proteins (RPP) class: tetO, tetQ, and tetW; Efflux class: tetB, tetH, and tetZ, refer to Levy et al. (1999) for the details on nomenclature) copy numbers were measured by quantitative PCR (qPCR) using the primers described in Table 2. The 16S rRNA genes and tetracycline resistance gene abundances were quantified against a standard curve generated from a plasmid containing the copies of respective genes, using a 10-fold serial dilution. The 20-µl qPCR mixtures contained 10 µl of 2× SYBR Green PCR Master Mix (Applied BioSystems, Foster City, CA, USA), 1.0 µl each of the 10 µM forward and reverse primers, and 7.0 µl of sterile, DNA-free water. Standard and environmental (ca. 1.0 ng) DNA samples were added at 1.0 µl per reaction. The reaction was carried out on an ABI Prism 7300 sequence detector (Applied Biosystems, Foster City, CA, USA) with an initial step of 95 °C for 10 min, followed by 40 cycles of denaturation (95 °C for 10 s), and primer annealing and extension (60 °C for 1 min). Dissociation curve analyses were performed to ensure the specificity of PCR, which included an increment of temperature

from 60 to 95 °C, at an interval of 0.5 °C for 5 s. Gene copy numbers were determined using a regression equation for each assay and relating the cycle threshold (CT) value to the known numbers of copies in the standards. The efficiencies of the qPCR were 90 to 95 % ( $R^2 > 0.991$ ). All qPCR reactions were run in quadruplicate with the DNA extracted from each sample.

### **PCR amplification and pyrosequencing**

The extracted DNA was amplified using primers targeting the V1 to V3 hypervariable regions of the bacterial 16S rRNA gene (Unno *et al.* 2010). The primers used for bacteria were V1-9F: 5'-X-ACGAGTTTGATCMTGGCTCAG-3' and V3-541R: 5'-X-AC-WTTACCGCGGCTGCTGG-3' (where X bar code is uniquely designed for each bioaerosol sample, followed by a common linker AC). Polymerase chain reactions were carried out under the following conditions: initial denaturation at 94 °C for 5 min, followed by 10 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C to 55 °C with a touchdown program for 45 s, and elongation at 72 °C for 90 s. This was followed by an additional 20 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 45 s, and elongation at 72 °C for 90 s. The amplified products were purified using the QIAquick PCR purification kit (Qiagen, CA, USA). Amplicon pyrosequencing was performed at Beijing Genome Institute (BGI), Hong Kong, China, using 454/Roche GS-FLX Titanium instrument (Roche, NJ, USA).

**Table 2.** Q-PCR primers used to quantify the abundance of 16S rRNA genes and tetracycline resistance genes.

Primer names	Target gene	Sequence (5'-3')	Resistance mechanism
338F	16S rRNA	ACT CCT ACG GGA GGC AGC	Not applicable
519R	gene	GWA TTA CCG CGG CKG	
TetB-FW	<i>tetB</i>	TAC GTG AAT TTA TTG CTT CGG	Efflux pumps (Aminov <i>et al.</i> 2002)
TetB-RV		ATA CAG CAT CCA AAG CGC AC	
TetH-FW	<i>tetH</i>	CAG TGA AAA TTC ACT GGC AAC	
TetH-RV		ATC CAA AGT GTG GTT GAG AAT	
TetZ-FW	<i>tetZ</i>	CCT TCT CGA CCA GGT CGG	
TetZ-RV		ACC CAC AGC GTG TCC GTC	
TetO-FW	<i>tetO</i>	ACG GAR AGT TTA TTG TAT ACC	Ribosomal protection proteins (Aminov <i>et al.</i> 2001)
TetO-RV		TGG CGT ATC TAT AAT GTT GAC	
TetQ-FW	<i>tetQ</i>	AGA ATC TGC TGT TTG CCA GTG	
TetQ-RV		CGG AGT GTC AAT GAT ATT GCA	
TetW-FW	<i>tetW</i>	GAG AGC CTG CTA TAT GCC AGC	
TetW-RV		GGG CGT ATC CAC AAT GTT AAC	

### **Analysis of pyrosequencing data**

The sequence data obtained by pyrosequencing were processed using mothur software (Schloss *et al.* 2009). Sequences shorter than 200 nucleotides with homopolymers longer than 8 nucleotides and all reads containing ambiguous base calls or incorrect primer sequences were removed. Next, the sequences were arranged against a SILVA alignment ([http://www.mothur.org/wiki/Alignment\\_database](http://www.mothur.org/wiki/Alignment_database)). Putative chimeric sequences were detected and removed via the Chimera Uchime algorithm contained within mothur (Edgar *et al.* 2011). All taxonomic classification was performed using mothur's version of the Ribosomal Database Project (RDP) Bayesian classifier, using a RDP training dataset ([http://www.mothur.org/wiki/RDP\\_reference\\_files](http://www.mothur.org/wiki/RDP_reference_files)) normalized to contain six taxonomic levels for each sequence at 80 % Naïve Bayesian bootstrap cutoff with 1000 iterations. All sequence data are available under the NCBI SRA accession no. SRP044637.

### **Statistical processing and analysis of results**

The variation in 16S rRNA gene and tetracycline gene abundances in SCBs equipped with different manure removal systems was assessed using a oneway ANOVA with Tukey's post-hoc tests for each pairwise comparison. All samples were standardized by random subsampling to 435 sequences per sample using the sub.sample command (<http://www.mothur.org/wiki/Sub.sample>) in mothur. Operational taxonomic units (OTUs) (at 97 % similarity) and rarefaction values were calculated using the mothur platform. OTU-based community similarity data was first square-root transformed to build the Bray–Curtis distance matrix. Nonmetric multidimensional

scaling (NMDS) was used to visualize the Bray–Curtis distances of bacterial community across all samples using primer PRIMER v6 (Clarke & Gorley 2006). To look at the effect of different types of manure collection system and ventilation type on bacterial community composition, an analysis of similarity (ANOSIM) with pairwise Bray–Curtis distance was performed with 999 random permutations and statistical significance was considered at  $P < 0.05$ . One-way ANOVA with Tukey’s post-hoc tests were conducted to determine whether the manure collection system had a significant impact on the relative abundance of bacterial taxa.

### **2.1.3. Results and discussion**

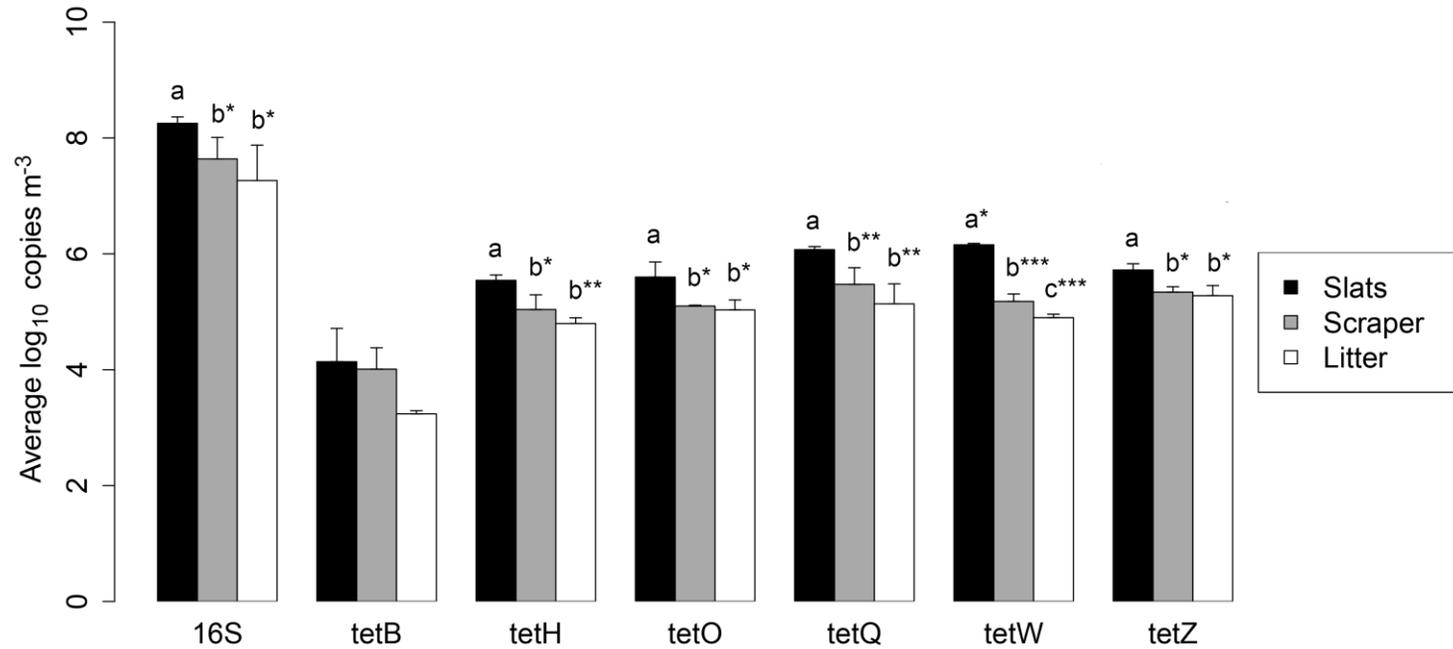
In this study, quantitative real-time PCR was used to evaluate the level of biotic contaminants and 454-pyrosequencing to characterize the bacterial bioaerosol community in SCBs. Pyrosequencing provided a comprehensive insight into the bacterial bioaerosols in SCBs.

#### **Effect of manure removal system on bacterial 16S rRNA and tetracycline gene abundances**

From the qPCR analyses, the bacterial 16S rRNA gene copy numbers differed in relation to manure removal system ( $P < 0.01$ ) (Figure 3), particularly between the deep-pit manure system with slats and manure removal system by scraper ( $P = 0.02$ ) and between deep-pit manure system with slats and deep-litter bed system ( $P = 0.01$ ). Airborne bacterial abundances peaked in bioaerosol samples collected from the SCBs equipped with deep-pit manure system with slats than

the scraper and litter systems (Figure 3). However, in a separate study involving different SCBs and culture-based methods, Kim *et al.* (2007) found that bacterial concentrations were highest in SCBs with a deep-litter bed system. The possible reasons for this discrepancy could be attributed to the difference in the techniques used to detect the bacterial concentrations and difference in the seasons of sample collection. Culture-independent methods are shown to be more accurate in determining the total airborne bacterial concentrations and estimate up to 1000 times higher than the concentration of airborne culturable bacteria (Nehme *et al.* 2008).

The abundance of tetracycline resistance genes were significantly higher in SCBs equipped with a deep-pit manure removal system with slats in comparison to scraper and deep-litter bed system, except for tetB gene (Figure 3). Swine manure is known to be a 'hot spot' of bacteria carrying tetracycline resistance genes (Heuer *et al.* 2002; Smalla *et al.* 2000), and the increased concentration of both 16S rRNA and tetracycline genes in bioaerosol samples of SCBs with the deep-pit system could be the product of longer storage of manure in the pits before removal.



**Figure 3.** Abundance of 16S rRNA and tetracycline resistance genes in swine confinement buildings equipped with different manure removal systems. Tukey pairwise comparisons are shown; different letters denote significant differences between groups. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$

## **Effect of manure removal system on bacterial community composition and diversity**

From 454-pyrosequencing, a total of 14,315 good quality bacterial sequences (with an average length of 309 bp) were obtained from the 8 samples, with an average of 1789 sequences per sample and with coverage ranging from 435 to 3013 reads per sample (Table 3). Of the 14,315 good-quality sequences, a total of 976 OTUs were obtained at  $\geq 97\%$  sequence similarity level. The average number of OTUs per sample was  $204 \pm 98$  (standard deviation [SD]), ranging from 84 to 344 OTUs (Figure 4).

The manure removal system was found to strongly influence the community composition and relative abundances of the major bacterial phyla in SCBs. The most abundant bacterial phylum was *Firmicutes* with 72.4 % of the sequences, followed by *Actinobacteria* (10.7 %), *Bacteroidetes* (9.8 %), and *Proteobacteria* (5.2 %) (Figure 5). Of these most abundant phyla, the relative abundance of *Firmicutes*, *Actinobacteria*, and *Proteobacteria* were found to be significantly different in SCBs with different manure removal systems (Figure 5). The relative abundance of *Firmicutes* was significantly higher ( $P=0.01$ ) in SCBs with slats and scraper than in deep-litter bed (Figure 5). *Actinobacteria* and *Proteobacteria* were found to be significantly abundant ( $P < 0.05$ ) in SCBs with deep-litter bed system compared to in the slats or scraper systems (Figure 5). The relative abundance of bacterial genera within the pre dominant phyla *Firmicutes* and *Actinobacteria* were further evaluated, and the bacterial bioaerosol community of SCBs was found to be predominantly made up by genera *Lactobacillus* (13.1 %), *Clostridium* (9.7 %), *Corynebacterium* (7.7 %),

*Staphylococcus* (3.3 %), and *Streptococcus* (2.7 %). Out of these, *Corynebacterium* was particularly enriched in SCBs with a deep-litter bed system ( $P < 0.01$ ) exhibiting an approximately 30-fold increase over SCBs with slats and scraper system.

The phylum *Firmicutes* has been shown to dominate SCB environments (Hong *et al.* 2012; Kumari & Choi 2014; Lee *et al.* 2006; Olson & Bark 1996). The predominant *Firmicutes* genera *Lactobacillus*, *Clostridium*, *Staphylococcus*, and *Streptococcus* are found to be commonly associated with the gastrointestinal tract of pig (Leser *et al.* 2002), suggesting that swine feces is the primary source of the phylum *Firmicutes* in the SCB bioaerosol. The higher relative abundance of the phylum *Firmicutes* in SCBs with the deep-pit system could be the result of longer storage of manure in the pits before removal. On the other hand, the dominance of *Actinobacteria* in the deep-litter bed system could be explained by the facts that they are capable to multiply in mixture of feces and litter in favorable temperature and aeration (Fries *et al.* 2005; Martin *et al.* 1998).

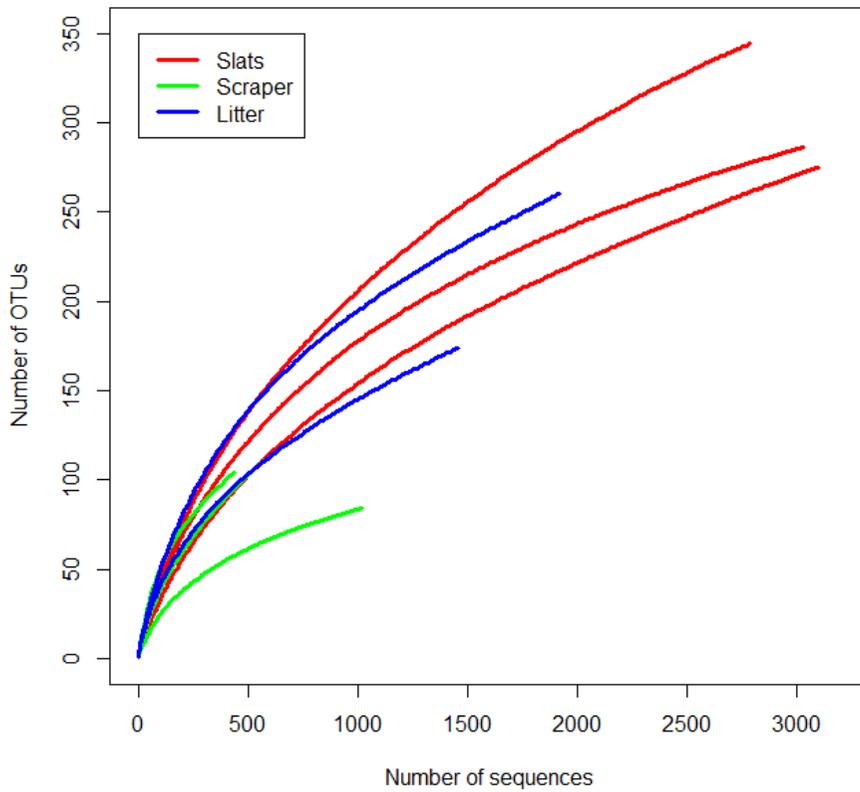
Bacterial diversity levels in bioaerosols were high in SCBs with deep-litter bed and deep pit with slats in comparison to scraper (Table 3). However, bacterial species-level richness (i.e., number of OTUs) did not differ among different manure removal systems ( $P = 0.27$ ). Similarly, none of the diversity indices differed between SCBs with different manure removal systems (Shannon  $P = 0.69$ , inverse Simpson  $P = 0.90$ , Chao  $P = 0.25$ , and Ace  $P = 0.36$ ). An NMDS plot of Bray–Curtis distance showed significant (ANOSIM:  $R = 0.68$ ,  $P < 0.01$ ) discrimination in composition of the airborne bacterial communities between samples collected from SCBs equipped with different manure

removal systems (Figure 6a), whereas there was no significant effect of air ventilation type on bacterial community composition (ANOSIM:  $R = -0.005$ ,  $P = 0.47$ ). The network analysis in Figure 6b highlights that bacterial communities in SCBs with the same manure removal system were more similar to each other than to communities of a different system with some overlap between slats and scraper systems. The network-based analysis complements the Bray–Curtis distance-based community analysis in Figure 6a, as the network was used to map the airborne bacterial community composition to specific manure removal systems. These results are in agreement with previous findings that a significant portion of the bioaerosols in confinement buildings originated from excreted feces and soiled bedding material (Duan *et al.* 2009; Dumas *et al.* 2011; Kumari & Choi 2014; Nehme *et al.* 2008; Nonnenmann *et al.* 2010). However, samples collected from SCBs with the scraper system did not cluster closely; one possible explanation for this could be that in the scraper system, manure can be removed from the swine house completely several times a day which results in a variation in bacterial community composition within the samples collected from the SCBs with the scraper system.

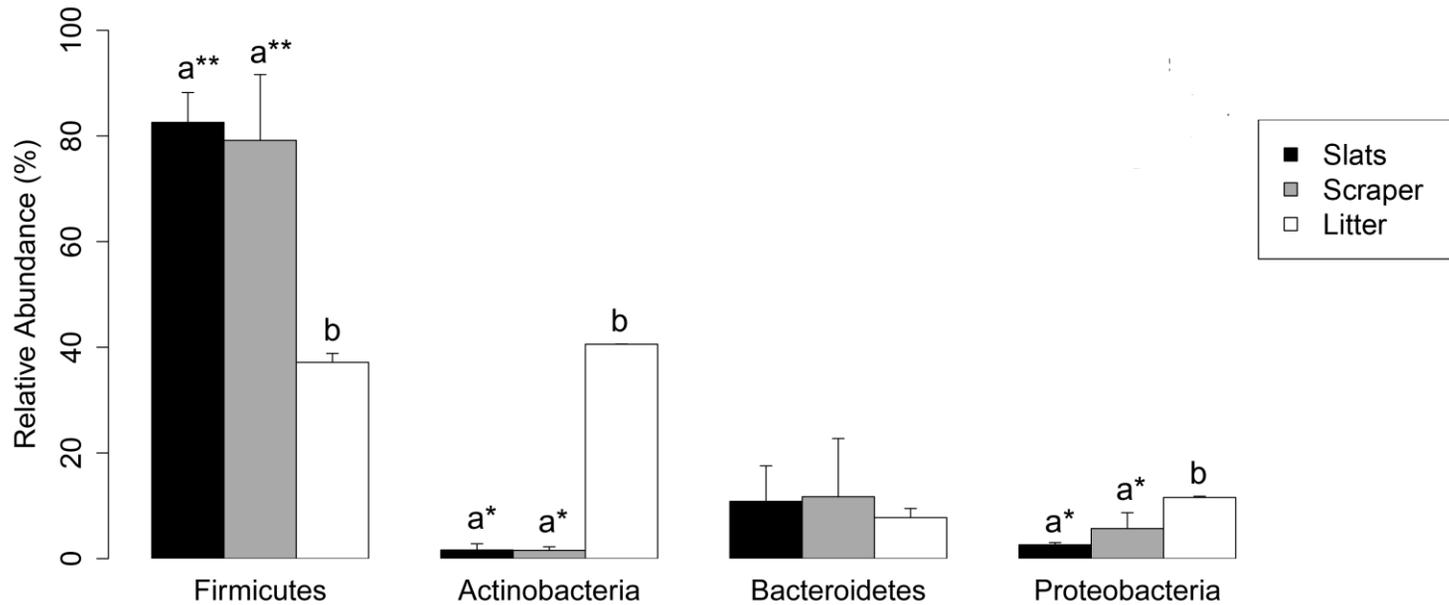
**Table 3.** Diversity indices of bioaerosol samples collected from swine confinement buildings.

ID	Manure removal system	Number of sequences	Normalized to 435 reads						
			OTU richness	PD <sup>a</sup>	Coverage	Shannon	Simpson	Ace	Chao
F1	Deep-pit manure system with slats	3028	119	7.624	0.8229	3.60	0.078	548.7	265.3
F2	Deep-pit manure system with slats	2787	118	7.682	0.8344	3.45	0.103	338.5	234.1
F3	Deep-pit manure system with slats	3103	100	6.814	0.8574	2.92	0.173	191.5	205.0
F4	Manure removal by scraper	573	96	6.490	0.8758	3.19	0.139	274.4	206.0
F5	Manure removal by scraper	1014	50	4.263	0.9448	2.05	0.310	103.4	80.6
F6	Manure removal by scraper	435	105	7.232	0.8919	4.03	0.029	190.8	188.1
F7	Deep-litter bed system	1917	128	8.601	0.8390	3.79	0.073	318.6	211.2
F8	Deep-litter bed system	1458	100	7.271	0.8850	3.46	0.084	170.9	158.3

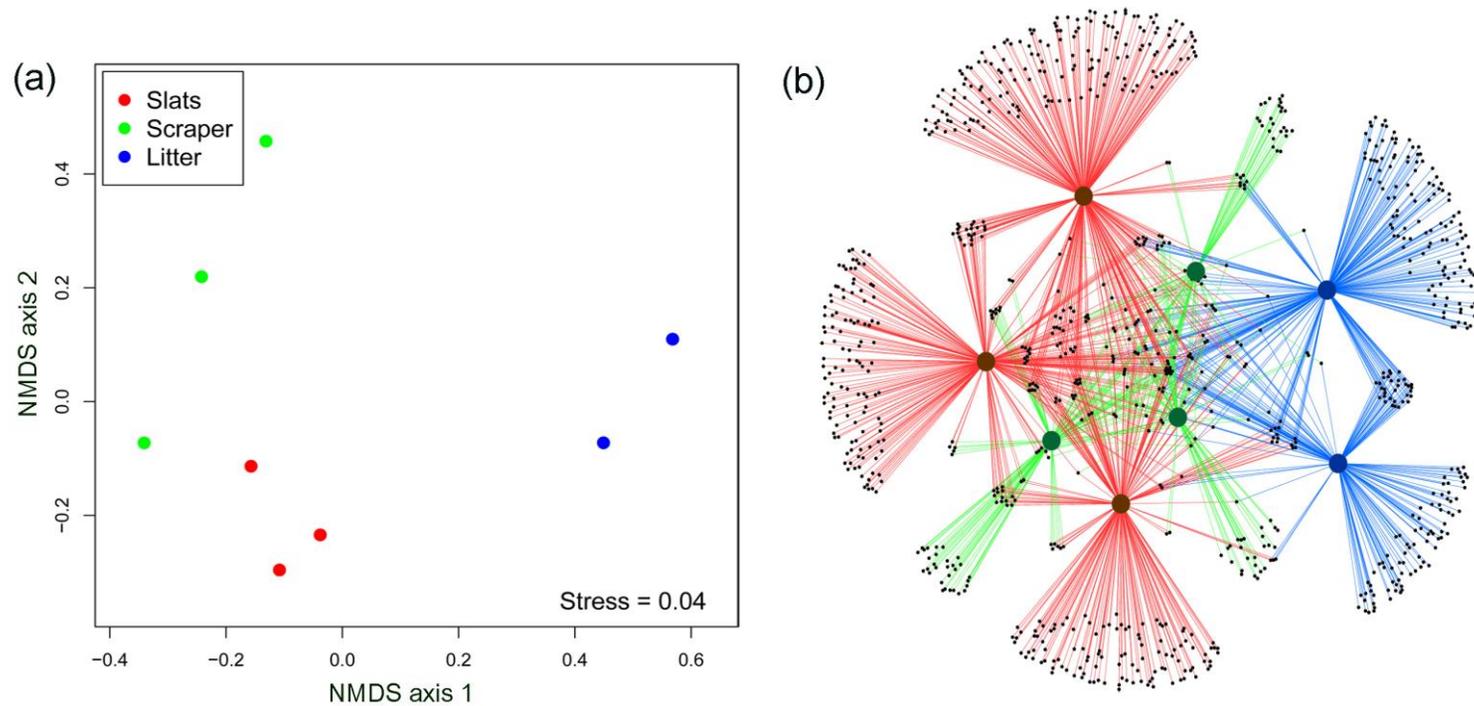
<sup>a</sup>PD: Phylogenetic diversity.



**Figure 4.** Rarefaction curves comparing airborne bacterial communities in swine confinement buildings with different manure removal systems.



**Figure 5.** Relative abundance (mean±SD) of dominant airborne bacterial phyla in swine confinement buildings equipped with different manure collection systems. Tukey pairwise comparisons are shown; different letters denote significant differences between groups. \* $P < 0.05$ ; \*\* $P = 0.01$



**Figure 6.** (a) NMDS plot of the pairwise Bray–Curtis distance matrix displaying OTU clustering of airborne bacterial communities in swine confinement buildings by manure removal systems. (b) Network analysis of the airborne bacterial communities in SCBs according to manure removal system.

#### **2.1.4. Conclusions**

In conclusion, the manure removal system strongly influenced the abundance and community composition of airborne biotic contaminants of SCBs. The airborne biotic contaminants were most abundant in SCBs equipped with a deep pit with slats. *Firmicutes* and *Actinomycetes* appear to be the dominant bacterial phyla in SCBs, and a comparison of relative abundances of these phyla together with dominant genera strongly supports the concept that individual bacterial lineages found in SCBs are enriched to specific manure removal systems. The present study represents a first glimpse of the airborne biotic contaminants of SCBs with different types of manure removal systems using next-generation sequencing methods. Based on the results of this study, better management practices and regulations can be designed to minimize the potential health impact on both livestock and humans working in this environment.

**CHAPTER 3. Seasonal  
Variability in Airborne Biotic  
Contaminants in Swine  
Confinement Buildings**

## **3.1. Seasonal variability in bacterial bioaerosols and antibiotic resistant genes in swine confinement buildings**

### **3.1.1. Introduction**

The intensification of pig farming in confined buildings with high animal densities can lead to poor indoor air quality. Microbial decomposition of proteinaceous waste products in feces and urine results in elevated concentrations of volatile organic compounds, NH<sub>3</sub>, and sulfides (O'Neill & Phillips 1992), whereas feed materials, skin debris, bedding material, and dried manure generate airborne particulates that carry adsorbed microorganisms and endotoxins (Cambra-López *et al.* 2010). Poor indoor air quality in SCBs affects both animal and human health. For example, airborne particulates can deposit in nasal channels and the respiratory tract and cause damage to lung tissues (Carpenter 1986). Furthermore, some airborne bacteria and gases such as NH<sub>3</sub>, H<sub>2</sub>S (from the manure), and CO<sub>2</sub> (pig activity) can cause or trigger chronic respiratory tract inflammation in workers and pigs (Charavaryamath & Singh 2006; Choudat *et al.* 1994; Cormier *et al.* 1990; Donham *et al.* 1989; Dosman *et al.* 2004; Heederik *et al.* 1991; Israel-Assayag & Cormier 2002; Mackiewicz 1998).

Microclimatic variables can influence the formation of aerosols containing microorganisms. There are great variations in the outside temperature in South Korea (-7 to 1°C in winter and from 22°C to 30°C in summer) (Korea 2008), so maintaining an optimal indoor temperature in SCBs can be challenging. Typically in the winter, all of the openings are closed, and the ventilation rate has to be minimal to

reduce the heat loss. This low ventilation rate could induce an increased concentration of airborne contaminants. In contrast, the ventilation is maximal during the summer, thus diminishing the indoor temperature and contributing to driving the indoor air outside the SCBs.

Bacteria constitute a huge proportion of organisms within bioaerosols in SCBs, with a mean concentration of  $10^5$  cfu m<sup>-3</sup> (Chang *et al.* 2001; Nehme *et al.* 2008). While much has been done to monitor the indoor airborne biotic contaminants in SCBs (Hong *et al.* 2012; Nehmé *et al.* 2009; Nehme *et al.* 2008), relatively little is known about the seasonal dynamics of airborne biotic contaminants and their interaction with microclimate parameters in SCBs. Nehme *et al.* (2009; 2008) examined the seasonal variability of airborne bacterial and archaeal communities in SCBs and found that, although the microbial abundances were significantly higher during the winter, the biodiversity was similar in each SCB during both the winter and summer seasons. However, these studies used low-resolution molecular fingerprinting tools, which lacked the coverage and depth of high-throughput sequencing methods. In a recent study, Hong *et al.* (2012) used 454-pyrosequencing to analyze the airborne biotic contaminants in pig and poultry confinement buildings sampled from different climate conditions and found that the different livestock as well as production phase were associated with distinct airborne bacterial communities; however, they did not evaluate the effect of microclimate variables on bacterial bioaerosol communities.

The usage of antibiotics in swine farms has promoted the development and abundance of antibiotic resistance in microbes (Blake *et al.* 2003; Zhu *et al.* 2013), which can become aerosolized within the

SCBs. Antibiotic resistant genes (ARGs) can be transferred to pathogens through transformation or phage-mediated transduction, and/or by conjugation, posing a serious threat to public health. Horizontal gene transfer plays important roles in the evolution and transmission of ARGs between bacterial species and includes the movement of ARGs from fecal bacteria to environmental bacteria, as well as the reverse; that is, emergence of novel mechanisms of acquired resistance in pathogens, ARGs that originally were present in harmless bacteria (Baquero *et al.* 2008). Tetracycline was chosen for this study because it is the most widely used broad spectrum antibiotic in livestock production worldwide, and is particularly prevalent in pig production (Delsol *et al.* 2003). The mechanism by which bacteria resist tetracycline antibiotics is heavily biased by ecological niche (Gibson *et al.* 2014), and compared to the existing literature on tetracycline resistance genes ( $Tc^R$ ) in soils and in water, relatively little is known regarding  $Tc^R$  genes in aerosols of SCBs (Hong *et al.* 2012; Ling *et al.* 2013). Three  $Tc^R$  genes (*tetB*, *tetH*, *tetZ*) encoding efflux proteins (EFP), and three others (*tetO*, *tetQ*, *tetW*) encoding ribosomal protection proteins (RPP) were selected for this study because these genes have been detected in aerosol of SCBs (Hong *et al.* 2012) and because these  $Tc^R$  genes encode two main mechanisms of bacterial resistance to tetracycline, which have been found associated with bacteria of public health interest (Chopra & Roberts 2001; Roberts *et al.* 2012; Santamaría *et al.* 2011).

Therefore, the aim of this study was to answer the following questions using the Illumina Hiseq sequencing of the V3 region of the 16S rRNA gene and qPCR of both 16S rRNA and  $Tc^R$  genes:

(1) How does the bacterial bioaerosol community composition and diversity vary in SCBs during the winter and summer seasons?

(2) Does the abundance of 16S rRNA and Tc<sup>R</sup> genes vary in SCBs during the winter and summer seasons?

(3) What are the major microclimate variables affecting the abundance, community composition, and diversity of airborne biotic contaminants in SCBs?

### **3.1.2. Materials and methods**

#### **Characteristics of animal confinement buildings**

The study was conducted in the winter (January) and summer (June) of 2013 on seven commercial pig farms located in six South Korean provinces. All the commercial pig farms sampled in this study are privately owned. Permission to access privately owned farms was given by the farm owners and for future permissions we can contact the owners. All samples were collected in the growing/finishing houses of SCBs. The average outdoor temperature across all of South Korea ranges from  $-7$  to  $1^{\circ}\text{C}$  in winter and  $22^{\circ}\text{C}$  to  $30^{\circ}\text{C}$  in summer. Temperature differences among the six provinces were less than  $2^{\circ}\text{C}$  (Korea 2008). Ventilation in SCBs was mechanical by exhaust fans on walls. The number of animals kept in each sampling room ranged from 140 to 480, and the stocking density varied from 0.88 to 1.41  $\text{m}^2/\text{head}$ . The age of the pigs varied from 67-150 days in each sampling room at the time of sampling. The manure removal system was deep-pit with slats, and the cleaning cycle varied from 4-6 months.

#### **Microclimate variables**

The microclimate variables were measured from three points in the aisle outside the pens at every 8 h interval till 24 h, and all the microclimate variables were reported as averages corresponding to each sampling period. Air temperature and relative humidity were measured with a hygrothermograph (SK-110TRH, SATO, Tokyo, Japan). Air speed was measured with an anemometer (model 6112, KANOMAX, Osaka, Japan). Particulate matter concentrations ( $\mu\text{g m}^{-3}$ ) were measured using an aerosol mass monitor (GT-331, SIBATA, Soka-city, Japan). The mass concentrations of PM10 (PM average aerodynamic diameter  $\leq 10 \mu\text{m}$ ), PM2.5 (PM mean aerodynamic diameter  $\leq 2.5 \mu\text{m}$ ), PM1 (PM mean aerodynamic diameter  $\leq 1 \mu\text{m}$ ), and total suspended particles (TSP) were obtained simultaneously, at a flow rate of  $2.83 \text{ L min}^{-1}$ . The concentrations of  $\text{NH}_3$ ,  $\text{H}_2\text{S}$  and  $\text{CO}_2$  were measured by gas detector tubes (Gastec Co., Ltd., Kanagawa, Japan).

### **Sample collection and DNA extraction**

Aerosol samples were collected from the middle point in the aisle outside the pens at a height of 1.4 m above the floor (Figure 2). Air samples were captured on sterile  $0.22\text{-}\mu\text{m}$  cellulose nitrate filters (Fisher Scientific, Pittsburgh, PA) via vacuum filtration with a flow rate of  $4 \text{ l min}^{-1}$  for 24 h. The cellulose nitrate filters were kept at  $4^\circ\text{C}$  until processing in the laboratory. Bacterial DNA was extracted directly from the filters using the PowerSoil DNA isolation kit (MoBio Laboratories, Carlsbad, CA). Individual filters were aseptically cut into small pieces, loaded into the bead tube of the DNA extraction kit, and heated to  $65^\circ\text{C}$  for 10 min followed by 2 min of vortexing. The remaining steps of the DNA extraction were performed according to

the manufacturer's instructions. The purified DNA was resuspended in 50 µl of solution S6 (MoBio Laboratories) and stored at -20°C until PCR amplification. Blank filters were analyzed alongside sample filters to test for contamination, and following DNA extraction and amplification, blank filters were consistently found to be free of microbial contaminants.

### **Illumina sequencing and data processing**

The extracted DNA was amplified using primers 338F (5'-XXXXXXXXX-GTACTCCTACGGGAGGCAGCAG-3') and 533R (5'-TTACCGCGGCTGCTGGCAC-3') targeting the V3 region of bacterial 16S rRNA ('X' denotes 8-mer barcode sequence) (Huse *et al.* 2008). Paired-end sequencing was performed at Beijing Genome Institute (BGI) (Hongkong, China) using 2 x 150 bp Hiseq2500 (Illumina) according to the manufacturer's instructions. Library preparation, sequencing, and initial quality filtering were performed as described previously (Zhou *et al.* 2011). The sequence data obtained by Illumina Hiseq2500 sequencing were processed using mothur (Schloss *et al.* 2009). Paired-end sequences were assembled, trimmed, and filtered in mothur. Next, the sequences were aligned against a SILVA alignment (<http://www.arb-silva.de/>). Putative chimeric sequences were detected and removed via the Chimera Uchime algorithm contained within mothur (Edgar *et al.* 2011). Sequences were denoised using the 'pre.cluster' command in mothur, which applies a pseudo-single linkage algorithm with the goal of removing sequences that are likely due to sequencing errors (Huse *et al.* 2010). All sequences were classified using the EzTaxon-e database ([43](http://eztaxon-</a></p></div><div data-bbox=)

e.ezbiocloud.net/) (Kim *et al.* 2012), using the classify command in mothur at 80% Naïve Bayesian bootstrap cutoff with 1000 iterations. Sequence data were deposited in SRA at NCBI with the accession number of SRP039383.

### **Quantification of 16S rRNA and Tc<sup>R</sup> genes**

A real-time polymerase chain reaction was used to quantify bacterial 16S rRNA and six Tc<sup>R</sup> genes (RPP class: *tetO*, *tetQ* and *tetW*; EFP class: *tetB*, *tetH* and *tetZ*, refer Levy et al. (1999) for the details on nomenclature) using the SYBR Green approach with the primers described in Table S1. The copy numbers of 16S rRNA and Tc<sup>R</sup> genes in bioaerosol samples were measured against the standard curves of plasmids containing copies of the respective genes, using a 10-fold serial dilution. All reactions were conducted in triplicate with the 20 µl qPCR mixtures containing 10 µl of 2X SYBR Green PCR Master Mix (Applied BioSystems, Foster City, CA, USA), 1.0 µl each of the 10 µM forward and reverse primers, and 7.0 µl of sterile, DNA-free water. Standard and bioaerosol (ca. 1.0 ng) DNA samples were added at 1.0 µl per reaction. The reaction was carried out on an ABI Prism 7300 sequence detector (Applied Biosystems, Foster City, CA, USA) with an initial step of 95°C for 10 min, followed by 40 cycles of denaturation (95°C for 10 s), and primer annealing and extension (60°C for 1 min). Dissociation curve analysis was performed to ensure the specificity of PCR, which included an increment of temperature from 60°C to 95°C at an interval of 0.5°C for 5 s. Gene copy numbers were determined using a regression equation for each assay and relating the cycle threshold (CT) value to the known numbers

of copies in the standards. The correlation coefficients of standard curves ranged from 0.957 to 0.996.

### **Statistical processing and analysis of results**

Rarefaction curves and diversity indices were generated using mothur, with the bacterial phylotype (OTU) defined here at 97% threshold of 16S rRNA gene sequence similarity. Phylogenetic diversity (PD) was calculated as Faith's PD (Faith 1992), the total phylogenetic branch length separating OTUs in each rarefied sample. To allow for robust comparisons among samples containing different numbers of sequences, phylotype richness and phylogenetic diversity were calculated based on samples rarefied to contain 15,909 sequences. To test for differences in relation to season on OTU richness, PD, 16S rRNA and Tc<sup>R</sup> genes abundances, we used a t-test for normal data and the Wilcoxon rank-sum test for non-normal data. We used the same procedure to test whether the relative abundance of the most abundant phyla differed across seasons.

To avoid including collinear variables in further analyses, we used a Spearman's correlation matrix and highly correlated (Spearman's  $r \geq 0.8$ ) microclimatic variables were removed from further analysis. Non-metric multidimensional scaling (NMDS) was generated based on pairwise Bray-Curtis dissimilarities between samples using the vegan R package (Oksanen *et al.* 2007). The analysis of similarity (ANOSIM) function in the vegan R package with 999 permutations was used to test for differences in bacterial communities among the winter and summer season. The vectors of microclimate variables were fitted onto ordination space (Bray-Curtis NMDS) to

detect possible associations between patterns of community structure and microclimate variables using the ‘envfit’ function of the vegan R package, and statistical significance was evaluated among 999 random permutations. Analyses for Venn diagram generation were performed using the mothur, and the Venn diagram was plotted using R package VennDiagram (Chen & Boutros 2011). Differentially abundant bacterial genera between the winter and summer seasons were identified using a parametric approach (Metastats) (White *et al.* 2009). All statistical analysis, graphs, and ordinations were produced using R version 3.0.2 (RDevelopmentCoreTeam 2008).

### **3.1.3. Results and discussion**

In this study, Illumina sequencing was used to provide a comprehensive insight into the bacterial bioaerosol community composition and diversity in SCBs. The use of high-throughput molecular sequencing methods revealed indoor microbial biodiversity that was previously difficult or impossible to observe (Sogin *et al.* 2006).

#### **Season variations in microclimate variables and bacterial bioaerosols diversity**

The means of microclimate variables in the SCBs during the winter and summer seasons are presented in Table 4. All of the microclimate variables in the SCBs differed significantly between the winter and summer season samples ( $P < 0.05$ ; Table 4), except total suspended particles,  $\text{NH}_3$  and  $\text{H}_2\text{S}$  ( $P > 0.05$ , Table 4). Spearman's correlation matrix showed highest correlation between PM10 and TSP

( $r = 0.93$ ; Table 5). Temperature and airspeed were the next most correlated variables ( $r = 0.9$ ; Table 5) followed by  $\text{NH}_3$  and  $\text{CO}_2$  ( $r = 0.82$ ; Table 2) and  $\text{PM}_{2.5}$  and  $\text{PM}_1$  ( $r = 0.81$ ; Table 5). Therefore, we removed  $\text{PM}_{10}$ , temperature,  $\text{PM}_1$  and  $\text{NH}_3$ , and used the remaining six microclimate variables, (i.e. airspeed, relative humidity,  $\text{PM}_{2.5}$ , TSP,  $\text{H}_2\text{S}$  and  $\text{CO}_2$ ) for further analyses.

From the 14 samples, we obtained 13,597 OTUs at 97% similarity from 497,607 good-quality sequences. The average number of OTUs per sample was  $2442 \pm 910$  (standard deviation [SD]), ranging from 1287 to 4045 OTUs. To compare diversity levels and community profiles between samples controlling for differences in sequencing depth, samples were compared at the same sequencing depth (15,909 randomly selected sequences per sample). At this depth of coverage, bacterial richness ( $P < 0.01$ ; Figure 7a) and phylogenetic diversity levels ( $P = 0.01$ ; Figure 7b) were significantly higher in winter in comparison to summer. This result is in contrast with the findings of Nehme et al.(2008), who examined the influence of seasonal variation on bacterial biodiversity in SCBs and found that biodiversity was unchanged between different seasons of the year. One of the possible explanations for this discrepancy in results could be that Nehme et al. (2008) analyzed very limited number of sequences for estimating the bacterial bioaerosol diversity. Furthermore, Nehme et al. (2008) used denaturing gradient gel electrophoresis and 16S-cloning-and-sequencing approach to characterize the bacterial bioaerosol community, which lack resolution and throughput, respectively, compared to next-generation sequencing based methods (Bartram *et al.* 2011; Bent *et al.* 2007; MacLean *et al.* 2009). Spearman's correlation

coefficients showed a significant negative correlation between air speed and both OTU richness and PD of bacterial bioaerosol communities (Table 6). Whereas, PM<sub>2.5</sub> and TSP were positively correlated to OTU richness and PD (Table 6). Airspeed has been shown to impact the diversity of indoor bacterial communities (Kembel *et al.* 2014), and these results suggest that the higher diversity levels in the winter season are likely to be a function of ventilation rate and particulate matter, as during summer under high ventilation rates, more airborne particulate matter carrying bacteria would be transferred out of the SCBs.

**Table 4.** Seasonal means ( $\pm$ SD) of microclimate variables in swine confinement buildings.

<b>Variable</b>	<b>Temp., °C</b>	<b>Relative humidity , %</b>	<b>Air speed, m/s</b>	<b>PM10, <math>\mu\text{g m}^{-3}</math></b>	<b>PM2.5, <math>\mu\text{g m}^{-3}</math></b>	<b>PM1, <math>\mu\text{g m}^{-3}</math></b>	<b>TSP, <math>\mu\text{g m}^{-3}</math></b>	<b>NH<sub>3</sub>, mg/L</b>	<b>H<sub>2</sub>S, mg/L</b>	<b>CO<sub>2</sub>, mg/L</b>
<b>Season</b>										
Winter	20.8 $\pm$ 5.5	68.5 $\pm$ 18.6	0.02 $\pm$ 0.02	470.8 $\pm$ 468.8	48.4 $\pm$ 27	25.7 $\pm$ 20.1	1252.2 $\pm$ 1320.5	24.4 $\pm$ 22.7	0.36 $\pm$ 0.34	2833.1 $\pm$ 1433.6
Summer	31.5 $\pm$ 4.2	86.3 $\pm$ 13.7	0.18 $\pm$ 0.06	77.5 $\pm$ 19.5	17.1 $\pm$ 8.2	7.6 $\pm$ 4.2	130.8 $\pm$ 34.2	14.9 $\pm$ 12.1	0.41 $\pm$ 0.43	1242.6 $\pm$ 423.7
<i>P</i> -value <sup>a</sup>	< 0.001	0.003	< 0.001	0.04	0.03	0.03	0.07	0.21	0.82	0.03

<sup>a</sup>For each variable, *P*-value was used to determine the significance of means among the two seasons.

**Table 5.** Spearman rank correlations between measured microclimatic variables in SCBs.

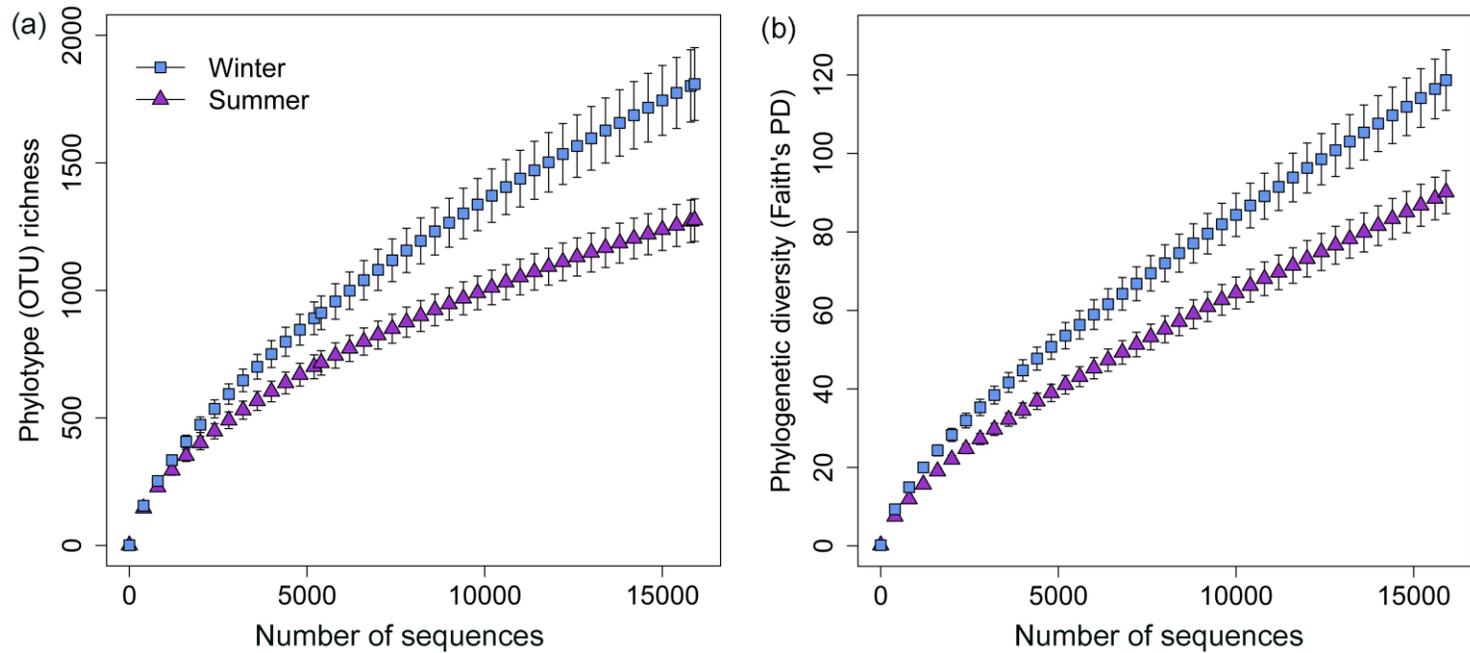
<b>Microclimate variables</b>	<b>Temp.</b>	<b>Relative humidity</b>	<b>Air speed</b>	<b>PM10</b>	<b>PM2.5</b>	<b>PM1</b>	<b>TSP</b>	<b>NH<sub>3</sub></b>	<b>H<sub>2</sub>S</b>	<b>CO<sub>2</sub></b>
Temp.	1									
Relative humidity	0.26	1								
Air speed	0.9***	0.3	1							
PM10	-0.52	-0.46	-0.39	1						
PM2.5	-0.69**	-0.35	-0.7**	0.72**	1					
PM1	-0.63*	-0.17	-0.64*	0.34	0.81***	1				
TSP	-0.39	-0.48	-0.23	0.93***	0.53	0.18	1			
NH <sub>3</sub>	-0.06	0.02	-0.28	-0.13	0.12	-0.02	-0.15	1		
H <sub>2</sub> S	0.21	-0.11	-0.06	-0.11	0.04	-0.11	-0.15	0.68**	1	
CO <sub>2</sub>	-0.26	-0.02	-0.46	0.19	0.48	0.3	0.14	0.82***	0.44	1

\* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

**Table 6.** Spearman rank correlations between microclimatic variables, diversity and abundances of airborne biotic contaminants in SCBs.

Microclimate variables	OTU richness	PD	16S rRNA gene	<i>tetB</i>	<i>tetH</i>	<i>tetZ</i>	<i>tetO</i>	<i>tetQ</i>	<i>tetW</i>
Relative humidity	-0.56*	-0.52	-0.4	-0.24	-0.26	-0.24	-0.47	-0.3	-0.39
Air speed	-0.7**	-0.71**	-0.78***	-0.14	-0.69**	-0.52	-0.61*	-0.68**	-0.73**
PM2.5	0.57*	0.57*	0.78***	0.24	0.62*	0.66*	0.58*	0.79***	0.79***
TSP	0.08	0.06	0.55*	0.45	0.56*	0.57*	0.64*	0.58*	0.6*
H <sub>2</sub> S	0.35	0.31	0.09	-0.11	-0.25	0.1	-0.19	-0.03	-0.07
CO <sub>2</sub>	0.42	0.4	0.56*	-0.24	0.32	0.63*	0.41	0.6*	0.49

\* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .



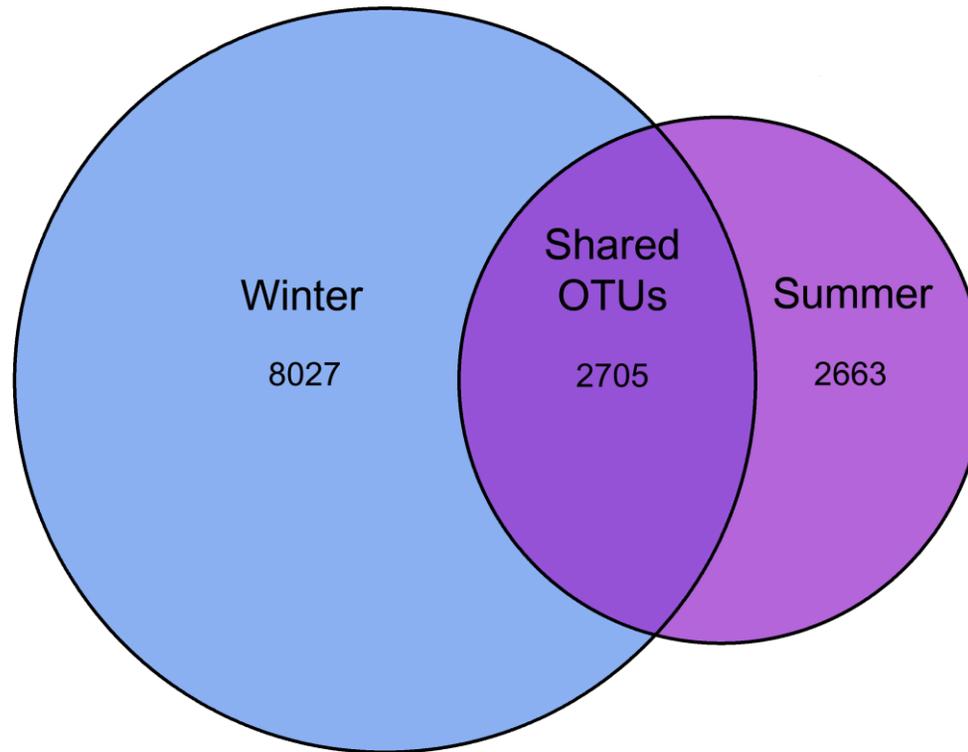
**Figure 7.** Rarefaction curves describing the bacterial (a) OTU richness and (b) phylogenetic diversity observed in the bioaerosol of SCBs during the winter and summer seasons. Diversity indices were calculated using random selections of 15,909 sequences per sample and error bars represent +1 s.e.m.

## **Effect of season and microclimatic parameters on community composition of bacterial bioaerosols**

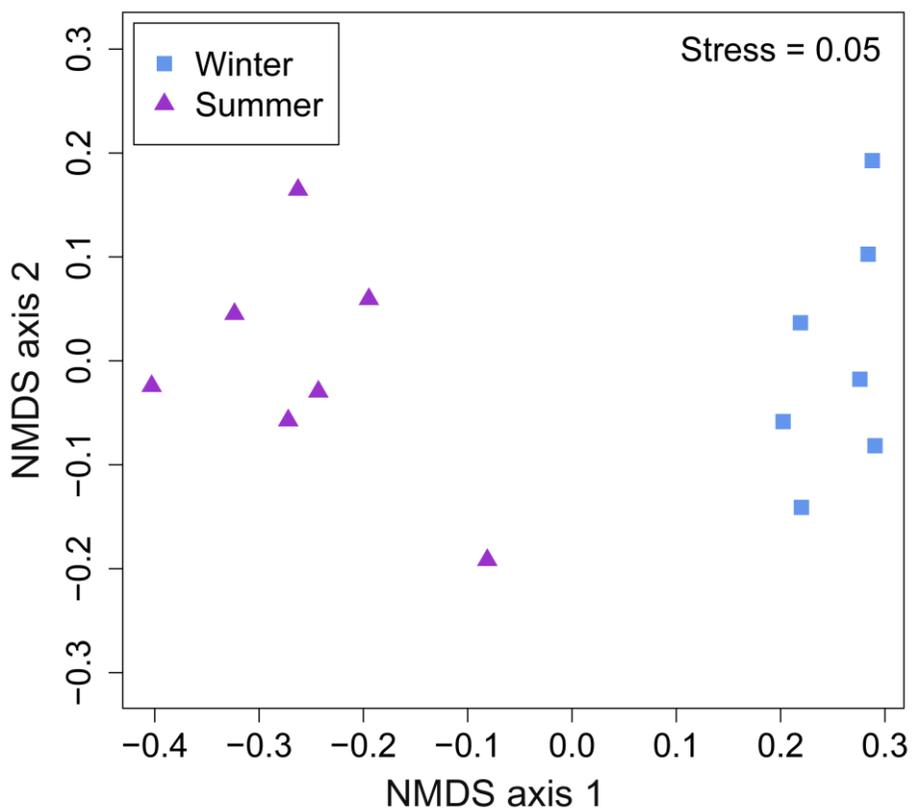
Venn diagrams (Figure 8) illustrate that OTU overlap between seasons as well as show unique OTUs. Overall, 20% of OTUs were shared between the winter and summer season. These 2,705 shared OTUs represented the majority of sequences (457,100 sequences or 91% of the total data set) (Figure 8). The OTU composition of the airborne bacterial communities was significantly influenced by seasons (ANOSIM statistic  $R = 0.96$ ,  $P < 0.01$ ; Figure 9). The samples collected during the winter season harbored bacterial communities distinct from those found in samples collected during summer. An environmental fitting analysis, using microclimatic variables, showed that airspeed ( $r^2 = 0.70$ ,  $P = 0.002$ ), PM<sub>2.5</sub> ( $r^2 = 0.39$ ,  $P = 0.04$ ), TSP ( $r^2 = 0.39$ ,  $P = 0.04$ ) and CO<sub>2</sub> ( $r^2 = 0.43$ ,  $P = 0.04$ ) were significantly associated with bacterial community composition. In several previous studies, it has been reported that microclimate variables are the most important factor which affects the indoor bacterial bioaerosol community composition and diversity (Kembel *et al.* 2012; Kembel *et al.* 2014). This relationship could be due to a direct link between the growth and survival of certain taxa and microclimate conditions in SCBs, or an increase in the dispersal of microbes from animals or animal feces under these conditions.

The most abundant bacterial phyla were *Firmicutes*, representing 50.9% of all sequences, followed by *Bacteroidetes* (21.3%), *Proteobacteria* (18.5%), and, to a lesser degree, *Actinobacteria* (3.9%), *Tenericutes* (0.6%), and *Spirochaetes* (0.4%); 0.8% of all sequences were unclassified. The phylum distribution

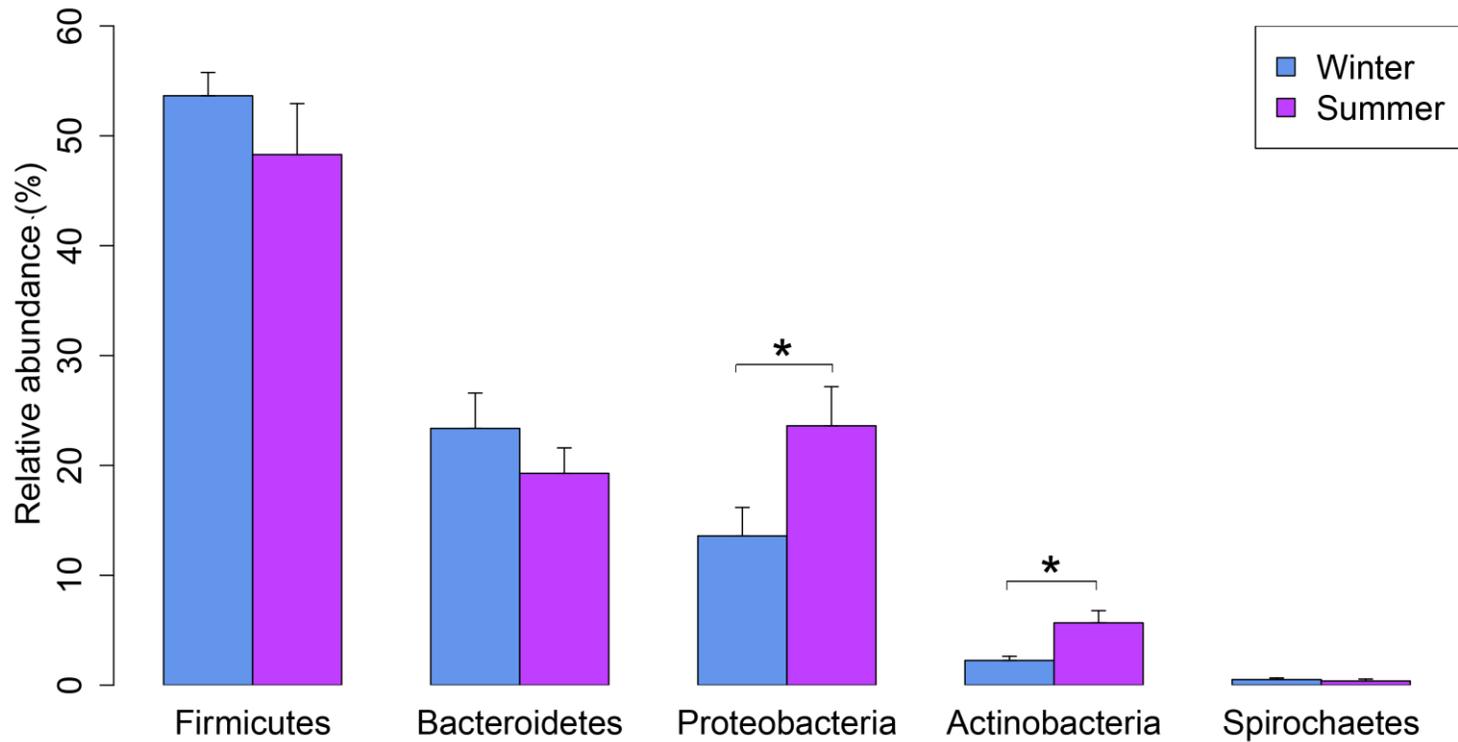
observed in this study in bioaerosol samples of SCBs is consistent with previous studies (Hong *et al.* 2012; Kristiansen *et al.* 2012; Nehme *et al.* 2008). We found significant differences in relative abundance across seasons for *Proteobacteria* ( $P = 0.04$ ) (Figure 10) and *Actinobacteria* ( $P = 0.03$ ) (Figure 10). We investigated in more detail what bacterial genera determine more strongly the distinct community composition in each season. Some bacterial genera were found to be dominant in both winter and summer seasons. *Lactobacillus* (18.3% and 16.3% on average) and *Prevotella* (19.6% and 6.2%) were the most dominant genera in both seasons. There were also some differences in abundant genera between the two seasons (Table 7). The bacterial bioaerosol of SCB in the winter season was dominated by a single genus – *Prevotella* – at nearly 19.6% (Metastats  $P = 0.01$ ). *Faecalibacterium* (0.8%), *Blutia* (0.7%), and *Catenibacterium* (0.7%) were also more abundant in the winter than in the summer (all  $P \leq 0.01$ ). Genera that were more abundant in the summer included *Sphingomonas* (3.7%), *Capnocytophaga* (3%), *Haemophilus* (2.8%), and *Streptococcus* (2.6%) (all  $P \leq 0.01$ ). The seasonal difference in the relative abundance of several bacterial genera justifying the view that the bacterial bioaerosol communities in both the winter and summer season samples are different.



**Figure 8.** Venn diagrams showing the overlap of OTUs (at 97% similarity) between winter and summer seasons. All samples in each season were pooled and then the percentage of shared and season-specific OTUs was calculated.



**Figure 9.** NMDS of Bray-Curtis pairwise dissimilarity of bacterial bioaerosol community in SCBs during the winter and summer seasons.



**Figure 10.** Relative abundance (means ± SE) of the most abundant bacterial phyla detected in SCBs bioaerosol during the winter and summer seasons. \* indicates significantly different at .05.

**Table 7.** Differentially abundant bacterial genera in swine confinement buildings sampled during winter and summer seasons. Differences in relative abundance of bacterial genera between seasons are represented with Metastats p-values. Significantly different ( $P \leq 0.01$ ) and relatively abundant genera ( $> 0.3\%$ ) were displayed.

<b>Phylum</b>	<b>Genus</b>	<b>Winter (%)</b>	<b>Summer (%)</b>	<b>P-value</b>
<i>Bacteroidetes</i>	<i>Capnocytophaga</i>	0.00	<b>3.06</b>	< 0.01
<i>Bacteroidetes</i>	<i>Chitinophaga</i>	0.01	<b>0.68</b>	< 0.01
<i>Bacteroidetes</i>	<i>Dyadobacter</i>	0.00	<b>0.37</b>	< 0.01
<i>Bacteroidetes</i>	<i>Prevotella</i>	<b>19.57</b>	6.24	0.01
<i>Firmicutes</i>	<i>Blautia</i>	<b>0.75</b>	0.19	0.01
<i>Firmicutes</i>	<i>Bulleidia</i>	<b>0.56</b>	0.33	< 0.01
<i>Firmicutes</i>	<i>Butyricicoccus</i>	<b>0.49</b>	0.19	0.01
<i>Firmicutes</i>	<i>Catenibacterium</i>	<b>0.74</b>	0.28	< 0.01
<i>Firmicutes</i>	<i>Faecalibacterium</i>	<b>0.87</b>	0.20	< 0.01
<i>Firmicutes</i>	<i>Oscillibacter</i>	<b>0.38</b>	0.12	0.01
<i>Firmicutes</i>	<i>Ruminococcus</i>	<b>0.33</b>	0.11	< 0.01
<i>Firmicutes</i>	<i>Streptococcus</i>	0.37	<b>2.64</b>	0.01
<i>Firmicutes</i>	<i>Subdoligranulum</i>	<b>0.69</b>	0.28	0.01
<i>Proteobacteria</i>	<i>Escherichia</i>	0.02	<b>1.26</b>	< 0.01
<i>Proteobacteria</i>	<i>Haemophilus</i>	0.00	<b>2.82</b>	< 0.01
<i>Proteobacteria</i>	<i>Neisseria</i>	0.00	<b>0.32</b>	0.01
<i>Proteobacteria</i>	<i>Sphingomonas</i>	0.17	<b>3.70</b>	0.01

## Comparison of aerial and fecal bacterial community

The bacterial bioaerosol community was compared with fecal bacterial community to see the similarity and differences in aerial and fecal bacterial community. At the phylum level both aerosol and fecal samples had very similar composition (Figure 11a). There were some differences in relative abundance of dominant bacterial genera in aerial and fecal samples (Figure 11b), however these dominant genera were present in both aerial and fecal samples (Figure 11b). Venn diagram was used to illustrate the overlap of bacterial OTUs between aerial and fecal samples. Overall, 39% of bacterial OTUs were shared between the aerial and fecal samples. These 1,242 shared bacteria OTUs represented the majority of sequences (90.8% of the total sequences) (Figure 12). These results validate the previous claims that swine feces are the primary source of the bacteria in the bioaerosols of SCBs (Hong *et al.* 2012; Nehme *et al.* 2008; Nonnenmann *et al.* 2010).

## Seasonal variations in abundance of 16S rRNA and Tc<sup>R</sup> genes

The abundances of 16S rRNA genes in the bioaerosols of SCBs were significantly higher in the winter (mean =  $1.4 \times 10^8$  bacteria  $\text{m}^{-3}$ ,  $n = 7$ ,  $P < 0.01$ , Figure 13) than in the summer (mean =  $1.2 \times 10^7$  bacteria  $\text{m}^{-3}$ ,  $P < 0.01$ ,  $n = 7$ , Figure 13). Six classes of Tc<sup>R</sup> genes (*tetB*, *tetH*, *tetZ*, *tetO*, *tetQ*, and *tetW*) were further quantified using qPCR. All six classes of Tc<sup>R</sup> genes were detected in high abundance in both winter and summer bioaerosol samples (Figure 13). Tc<sup>R</sup> genes encoding RPP (*tetO*, *tetQ* and *tetW*) were present in significantly higher copy numbers than Tc<sup>R</sup> encoding EFP (*tetB*, *tetH* and *tetZ*; *t*-test,  $P$ -value = 0.04). Seasonal trends were also detected in four Tc<sup>R</sup> genes,

which include *tetH*, *tetO*, *tetQ*, and *tetW*, and their abundances peaked in bioaerosol samples collected during the winter (Figure 13). The high abundance of 16S rRNA and Tc<sup>R</sup> genes detected in the bioaerosol samples suggests an alternative airborne transmission route, which can lead to their persistence and dispersal into the nearby environment. The level of contamination detected in this study was far higher than the proposed limit of bacterial contamination associated with human respiratory symptoms (Cormier *et al.* 1990). Similar to several previous studies, our results indicate that swine workers are exposed to a higher level of airborne bacteria than the occupational recommendations (Chang *et al.* 2001; Kristiansen *et al.* 2012; Nehme *et al.* 2008). Seasonal trends in microbial 16S rRNA gene concentration have already been reported by Nehme *et al.* (2009; 2008), who showed that total microbial 16S rRNA genes concentrations in bioaerosol of SCBs were significantly higher in winter. Seasonal fluctuation in Tc<sup>R</sup> genes abundances have been reported several times in wastewater treatment plants and livestock lagoons (McKinney *et al.* 2010; Yang *et al.* 2013); however, this is the first time a seasonal trend has been reported in bioaerosol samples of SCBs.

The prevalence of Tc<sup>R</sup> genes encode RPP in the present study is not surprising, since these genes were found to be predominant in the gastrointestinal tracts of pigs and steers (Aminov *et al.* 2001), and their elevated possibilities of transfer from one bacteria to another because of their close relationship with mobile genetic elements such as plasmids, conjugative transposons, integrons, and consequently their wide host range (Chopra & Roberts 2001; Roberts *et al.* 2012). Among all Tc<sup>R</sup> genes, *tetQ* had the highest abundance in bioaerosol samples (the

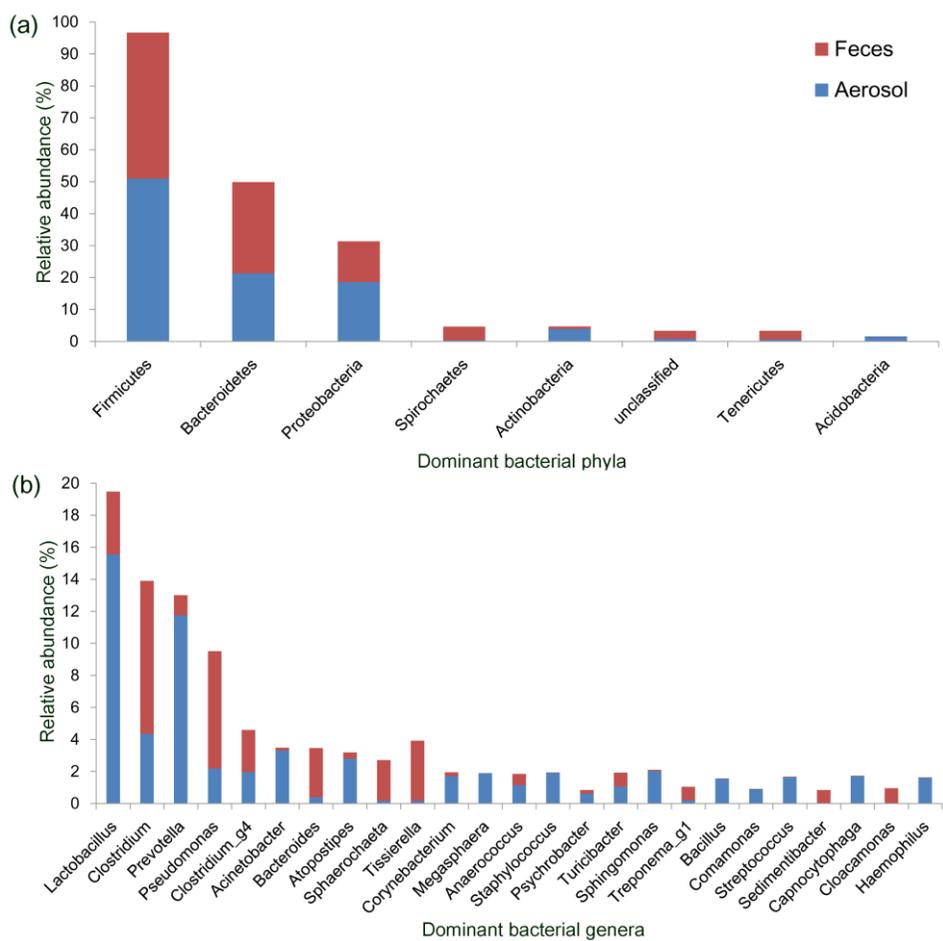
average abundance was  $8.89 \times 10^5 \pm 1.45 \times 10^6$  copies  $m^{-3}$ ) followed by *tetZ*, *tetO*, *tetW* and *tetH*, with *tetB* having the least abundance. The relatively high level of *tetQ* is not surprising because it is seen equally in both Gram-positive and Gram-negative bacterial genera (Roberts *et al.* 2012), and most of which have been shown to dominate the bioaerosols of SCBs such as *Clostridium*, *Lactobacillus*, *Staphylococcus*, *Streptococcus*, *Prevotella* etc. (Hong *et al.* 2012; Kristiansen *et al.* 2012; Nehme *et al.* 2008). The lack of *tetB* and *tetH* is also not surprising because they are found only associated with Gram-negative bacteria (Roberts *et al.* 2012), which are less common in bioaerosols of SCBs. However, higher average abundance of *tetZ* compared to *tetO* and *tetW* was not expected because it has relatively narrow host range (Only detected in *Lactobacillus* and *Corynebacterium*; (Roberts *et al.* 2012)). These results are consistent with the recent findings that both ecology and bacterial phylogeny are the primary determinant of ARG content in environment (Forsberg *et al.* 2014; Gibson *et al.* 2014). qPCR is more indicative of the potential for aerosol-mediated transfer of antibiotic resistance between environments than culture-based methods, and results can be more easily compared among studies. Nonetheless, the method is limited by its ability to detect only a fragment of genes targeted. Truncated sequences and non-expressed sequences cannot be resolved from expressed gene sequences using qPCR, so the levels reported could overestimate the number of functional, full length genes.

Among the six microclimate variables, air speed was found negatively correlated ( $P < 0.05$ ) with the abundances of 16S rRNA, *tetH*, *tetO*, *tetQ*, and *tetW* genes (Table 3), however, PM<sub>2.5</sub> and TSP

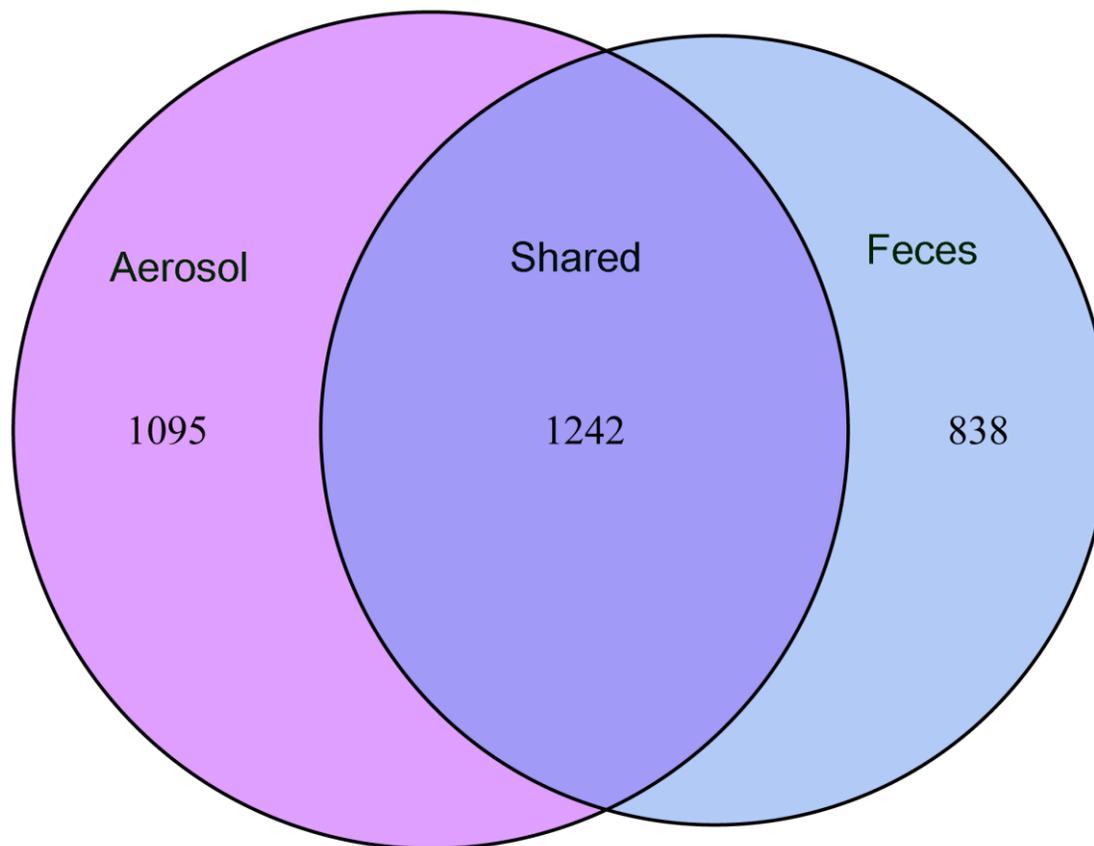
were found positively correlated ( $P < 0.05$ ) with these genes (Table 6). The reduced ventilation in SCBs to avoid heat loss in winter could be responsible for the increased concentration of TSP and PM<sub>2.5</sub> in SCBs, which in turn could increase the abundance of 16S rRNA and Tc<sup>R</sup> genes. In this survey only a limited number of ARGs were investigated in bioaerosols, however, a variety of ARGs encoding different antibiotic resistance could be present in bioaerosols of SCBs, where different classes of antibiotics (e.g., macrolides, lincosamides) are frequently used in addition to tetracycline. So there is a need for further study to explore more diverse ARGs in bioaerosols of SCBs.

The detection of 16S rRNA and Tc<sup>R</sup> genes in high abundance is of particular concern, because Tc<sup>R</sup> airborne pathogenic bacteria present in SCBs have been shown to colonize the nasal flora of pig farmers (Létourneau *et al.* 2010), and this could pose potential occupational health problem. Indoor air ventilated from the SCBs to the external environment can cause detrimental effects to the ambient air quality. For instance, multiple drug resistances bacteria were recovered in an air plume up to 150 m downwind from a SCB at higher percentages than upwind (Gibbs *et al.* 2006). Furthermore, presence of some airborne pathogens have been detected over long distances from their emission site which were found capable to infect healthy animals intramuscularly or intratracheally (Otake *et al.* 2010). Most of the previous studies consistently indicated an association between environmental exposure to SCBs and respiratory symptoms indicative of asthma of their neighbors (Kilburn 2012; Pavilonis *et al.* 2013; Schinasi *et al.* 2011). Surprisingly, Smit *et al.* (2013) found an inverse association between indicators of air pollution from livestock farms and

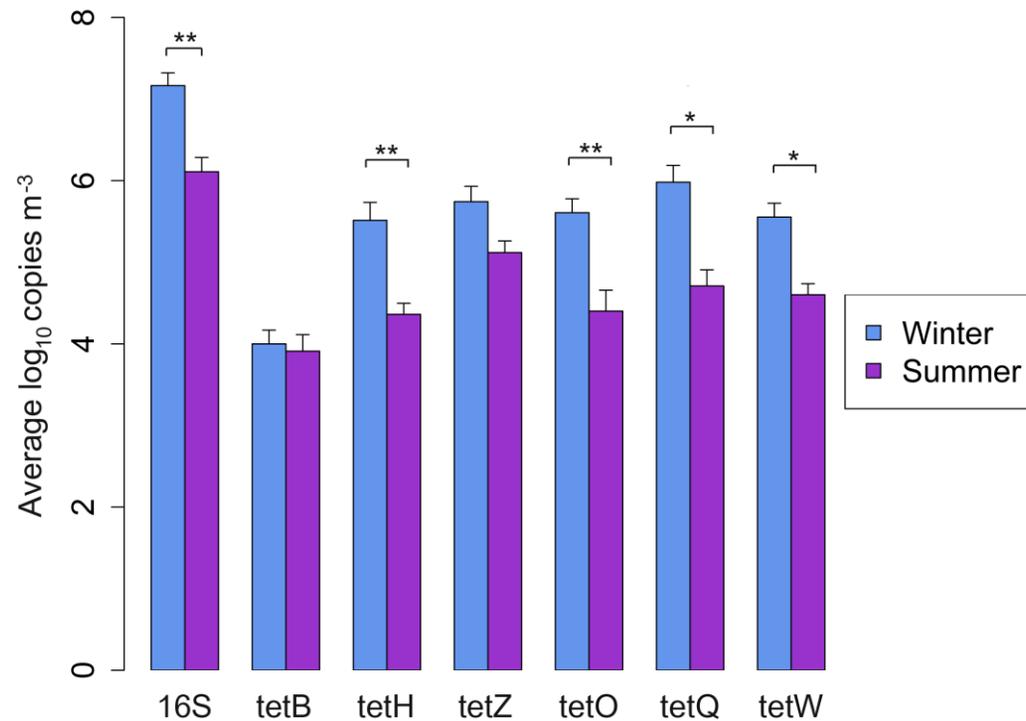
respiratory morbidity among neighboring resident. However, before drawing firm conclusions from this study, these results should be confirmed with more objective disease information.



**Figure 11.** Comparison of relative abundance of (a) dominant bacterial phyla and (b) dominant bacterial genera between aerial and fecal samples.



**Figure 12.** Venn diagrams showing the overlap of bacterial OTUs between aerosol and fecal samples.



**Figure 13.** Abundance of 16S rRNA and tetracycline resistance genes in the bioaerosols of SCBs. Asterisks above solid lines indicate significant differences between the winter and summer seasons samples of SCBs. \* indicates significantly different at  $< 0.05$ , \*\* indicates at  $< 0.01$ .

### **3.1.4. Conclusions**

Though, this study had fewer samples than previous studies, our results however indicate that seasons have an influence on the biotic contaminants abundance, community composition and diversity, in indoor air of SCBs. Seasonality was significantly associated with microclimate variables, indicating that indoor environmental conditions play an important role in structuring airborne biotic contaminants in SCBs. Based on the results of this study, better management practices and regulations can be designed to minimize the potential health impact on both the farm workers and the public residing in close proximity to these buildings.

## **3.2. Seasonal variations in community composition and the diversity of airborne fungi in swine confinement buildings**

### **3.2.1. Introduction**

Modern animal husbandry has changed in recent years from pasture-based animal farming to the use of confinement buildings with high animal density. The concentrations of volatile organic compounds, ammonia (NH<sub>3</sub>), sulfide and particulate matter are elevated in the indoor environment of confinement buildings due to the high animal density (Cambra-López *et al.* 2010; O'Neill & Phillips 1992), which leads to poor indoor air quality. The particulate matter contains fungal spores and endotoxins, which can cause lung infections and airway-related inflammatory responses in both farmers and animals (Auvermann *et al.* 2006; Bakutis *et al.* 2004; Radon *et al.* 2001).

Culture-based methods have been predominantly used in earlier studies of airborne fungi in various animal confinement buildings (Chang *et al.* 2001; Clark *et al.* 1983; Predicala *et al.* 2002). The fungal colony forming units (cfu) reported in these studies range in concentration from 10<sup>3</sup> cfu/m<sup>3</sup> to 10<sup>6</sup> cfu/m<sup>3</sup>, and *Cladosporium*, *Aspergillus* and *Penicillium* were detected as the predominant fungal genera. Other genera were detected, including *Alternaria*, *Fusarium*, *Verticillium*, and *Geotrichum*. It has also been found that indoor air fungal concentrations and emissions are influenced by the manure removal system in SCBs (Kim *et al.* 2008c). Viegas *et al.* (2013) studied air borne fungi in Portuguese swine farms and detected keratinophilic (*Scopulariopsis brevicaulis*) and toxigenic fungi

(*Aspergillus*, *Fusarium*, and *Penicillium* genera and *Stachybotrys chartarum*), suggesting a potential occupational health threat. Jo et al. (2005) studied airborne fungi concentrations in swine sheds and reported that the summer concentrations of total fungi and fungal genera inside the swine sheds were substantially higher than the winter values. A recent study of the fungal community of swine confinement facility aerosols using amplification of small subunit rRNA found *Aspergillus-Eurotium* as the quantitatively most important fungal group (Kristiansen *et al.* 2012). In another recent study, Boissy et al. (2014) used shotgun pyrosequencing metagenomic analyses of DNA from settled surface dusts from swine confinement facilities and grain elevators and found that the fungal species DNA reads were 30-fold lower in swine confinement facilities than in grain elevators. However, to the best of our knowledge, no molecular studies have investigated the seasonal trends of airborne fungal community composition and diversity in SCBs.

In this study, we collected aerosol samples from seven commercial swine farms in South Korea during the winter and summer seasons, and the fungal community composition and diversity were analyzed using the Illumina HiSeq sequencing platform. The objectives of this study were as follows:

(1) To determine the effect of season on airborne fungal community composition and diversity in SCBs.

(2) To identify the potential human allergen/pathogen related fungal genera present in SCBs.

(3) To study how their relative abundances vary between the winter and summer seasons.

### **3.2.2. Materials and methods**

#### **Aerosol collection**

Aerosol samples were collected from seven commercial swine farms in South Korea in winter (January) and summer (June) of 2013, with prior permissions from farm owners. Detailed characteristics of SCBs and the methods used to collect aerosol samples have been described in section 3.1.2. We collected aerosol samples at a height of 1.4 m above the ground from three points in SCBs. Sterile cellulose nitrate filters (0.22  $\mu\text{m}$ ; Fisher Scientific, Pittsburgh, PA) were used to collect the aerosol samples via filtration with a flow rate of 4 L  $\text{min}^{-1}$  for 24 h. After aerosol collection, the filters were immediately maintained at 4 °C until transport to the laboratory, where the samples were frozen at -20 °C.

#### **DNA extraction and PCR amplification**

The PowerSoil DNA isolation kit (MoBio Laboratories, Carlsbad, CA) was used to extract DNA from filters by following the initial processing methods as described in Kumari et al (2014). The purified DNA samples were stored at -20 °C until PCR amplification. We amplified the ITS1 region using primer pairs ITS1FI2, 5'-GAACCGCGGARGGATCA-3' (Schmidt *et al.* 2013) and ITS2, 5'-GAACCGCGGARGGATCA-3' (White *et al.* 1990).

#### **Sequencing and data processing**

The amplicons were sequenced at the Beijing Genome Institute (BGI) (Hong Kong, China) using 2×150 bp Hiseq2000 (Illumina, San

Diego, CA, USA) according to the manufacturer's instructions. Pandaseq software was used to assemble the paired-end sequences with default quality score value, i.e., 0.6 (Masella *et al.* 2012). Furthermore, ambiguous bases and homopolymers (> 8 bp) were removed from assembled sequences using mothur (Schloss *et al.* 2009). Chimeric sequences were removed using the 'chimera.uchime' command in mothur (Edgar *et al.* 2011). To remove the sampling bias, we used the 'sub.sample' command in mothur to randomly select a subset of 24,000 sequences from each. The standardized sequences were classified against a named fungal ITS sequences database (Nilsson *et al.* 2009) using BLASTn ver. 2.2.19 (Altschul *et al.* 1990). Next, a fungal taxonomic identification tool FHiTINGS (Dannemiller *et al.* 2014c) was used with BLASTn output files to sum and sort the taxonomic ranks from kingdom to species. The potential human allergen/pathogen related fungal taxa were identified using a list of known fungal allergen/pathogen related taxa (Yamamoto *et al.* 2012). Furthermore, the QIIME implementation of UCLUST (Edgar 2010) was used to assign operational taxonomic units (OTUs) with a threshold of 97% sequence similarity. All sequence data are deposited in the MG-RAST server (Meyer *et al.* 2008) under MG-RAST IDs 4633146.3–4633187.3.

### **Statistical analysis**

The seasonal differences in OTU richness, Shannon index and the relative abundance of the most abundant phyla were evaluated using t-test and Wilcoxon rank-sum test for normal and non-normal data, respectively. Differentially abundant fungal classes and genera between the winter and summer seasons were identified using a

parametric approach (Metastats) (White *et al.* 2009). The p-values were adjusted using Benjamini and Hochberg methods to account for multiple comparisons (Benjamini & Hochberg 1995). Adjusted p-values of less than 0.05 were considered statistically significant. The co-occurrence network of all of the detected fungal genera was constructed using CONET software by following the example tutorial (<http://psbweb05.psb.ugent.be/conet/microbialnetworks/conet.php>) (Faust *et al.* 2012) and visualized using Cytoscape (Smoot *et al.* 2011). The Bray-Curtis distance was used to calculate the OTU-based community dissimilarity (Magurran 2013). Non-metric multidimensional scaling (NMDS) was used to visualize the change in OTU composition across the seasons. The seasonal difference in community composition was tested using an analysis of similarity (ANOSIM) in the vegan R package (Oksanen *et al.* 2007). We also performed a permutational dispersion analysis to test whether beta diversity is significantly different between seasons by using the betadisper function in the vegan R package (Anderson 2006). All statistical analysis, graphs, and ordinations were produced using R version 3.0.2 (RDevelopmentCoreTeam 2008).

### **3.2.3. Results and discussion**

In this study, we used a high-throughput Illumina Miseq platform to extensively study the airborne fungal community composition and diversity in SCBs. While several studies have comprehensively investigated the airborne bacterial community composition and diversity in SCBs using next-generation sequencing (NGS) methods (Hong *et al.* 2012; Kumari & Choi 2014), relatively

little is known about the fungal community composition and diversity.

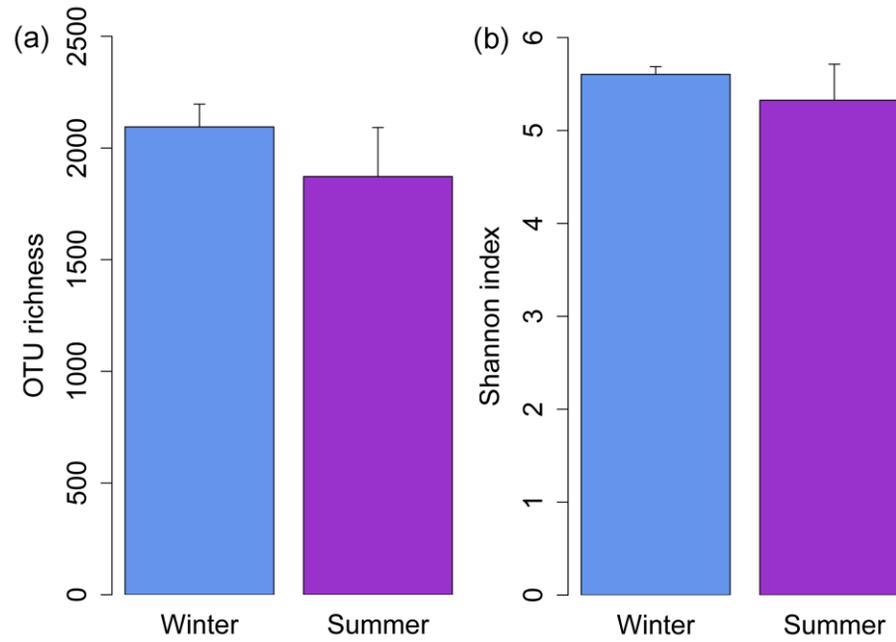
### **Effect of seasonal variations on fungal diversity**

From the 42 samples, we observed 22,399 OTUs at 97% sequence similarity from 1,008,000 good-quality sequences (24,000 randomly selected sequences per sample). The average number of the observed OTUs per sample was  $1,983 \pm 202$  (standard deviation [SD]), ranging from 1,524 to 2,345 OTUs. Similar to the bacterial diversity reported earlier in the same SCBs (Kumari & Choi 2014), the airborne fungal OTU richness and diversity were significantly higher in winter than in summer (Figure 14). Adams et al. (2013) also found higher fungal diversity in winter while studying the indoor air fungal community of residence buildings. One of the possible explanations of this high diversity could be that to maintain the indoor air temperature during winter, all of the openings are closed and the ventilation rates are reduced to minimal, which in turn increases the indoor bioaerosol particles and thus increases microbial diversity. The fungal richness and diversity information might be useful in terms of health and exposure evaluations of farmers working in SCBs, as fungal richness and diversity have been shown to be associated with asthma development (Dannemiller *et al.* 2014a; Ege *et al.* 2011).

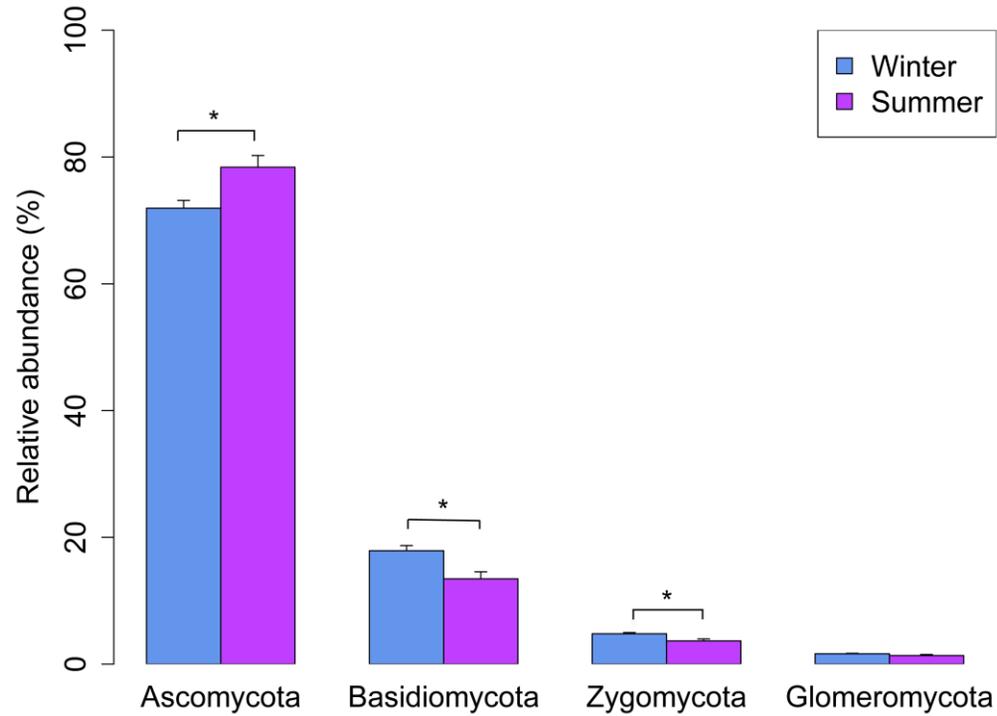
### **Effect of seasonal variations on fungal community composition**

The most abundant fungal phyla across all of samples were *Ascomycota*, representing 75.4% of all sequences, followed by *Basidiomycota* (15.3%), *Zygomycota* (4.2%), and *Glomeromycota* (1.5%) (Figure 15). The predominant fungal classes

detected in this study were *Dothideomycetes* and *Sordariomycetes* of phylum *Ascomycota*, which has been shown to dominate aerosol samples in several previous studies (Dannemiller *et al.* 2014b; Fröhlich-Nowoisky *et al.* 2009; Yamamoto *et al.* 2012). *Dothideomycetes* class is known to contain several allergenic fungal taxa (D'amato *et al.* 1997; Halonen *et al.* 1997; Shelton *et al.* 2002). *Agaricomycetes* was the most abundant class of phylum *Basidiomycota*, which does not generally contain described human allergen/pathogen related fungal taxa. The relative abundance of *Ascomycota* was significantly higher in summer ( $W=155$ ,  $P < 0.01$ ; Figure 15), whereas the relative abundances of *Basidiomycota* and *Zygomycota* were significantly higher in winter ( $W = 439$ ,  $P = 0.01$ ; Fig. 2). Comparisons between seasons were also conducted at the class and genus levels. After adjusting for multiple comparisons, the relative abundances of many classes and genera were significantly more abundant in a particular season (Table 8). The airborne fungal network contained 185 nodes (genera) and 1553 edges (lines connecting nodes) (Figure 16). Of these, 179 nodes and 1260 edges were positively associated, which means that they occur together and hence might share certain properties. Genus *Clavaria* was found to be connected to most of the positive edges (46 edges), whereas *Tomentella* connected to most of the negative edges (26 edges). The co-occurrence network analysis allowed us to identify the important relationships among fungal genera, which could be tested in the future under controlled conditions.



**Figure 14.** Seasonal variation in the fungal (a) OTU richness and (b) Shannon diversity index in swine confinement buildings.

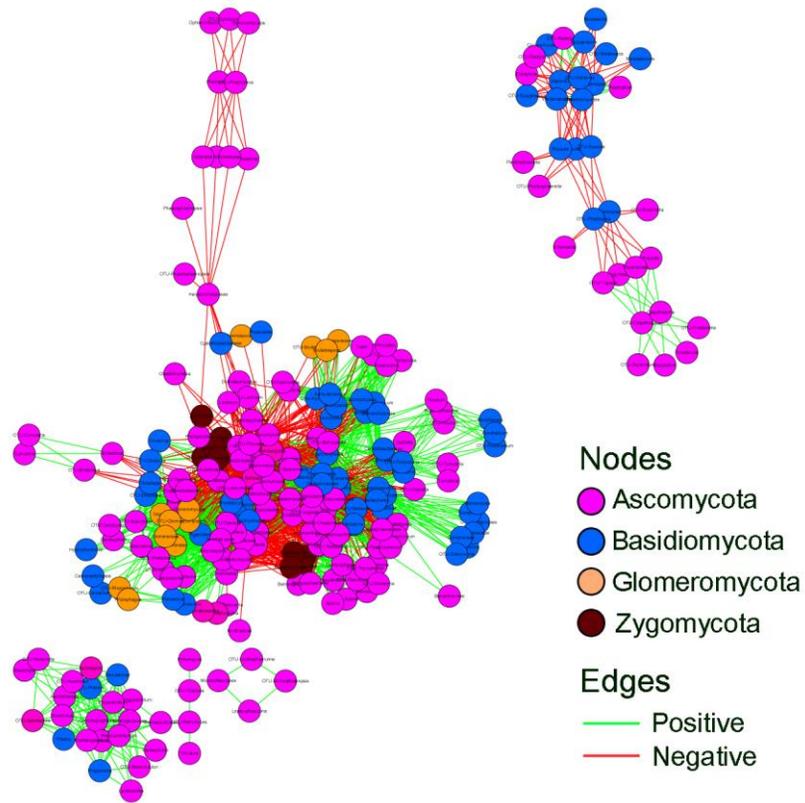


**Figure 15.** The relative abundance (means  $\pm$  SD) of the predominant fungal phyla of SCBs during the winter and summer seasons. The asterisk mark indicates significant differences between seasons.

**Table 8.** Differentially abundant fungal taxa in winter and summer seasons. The fungal taxa whose relative abundance was less than 0.01% across all samples were excluded. Only significantly different taxa ( $q < 0.05$ ) are displayed.

<b>Season</b>	<b>Class</b>	<b>Genus</b>
Winter	<i>Agaricomycetes</i>	<i>Trichosporon</i>
	<i>Pezizomycetes</i>	<i>Emericella</i>
	<i>Tremellomycetes</i>	<i>Calyptrozyma</i>
	<i>Saccharomycetes</i>	<i>Lophiostoma</i>
	<i>Pucciniomycetes</i>	<i>Devriesia</i>
		<i>Massarina</i>
		<i>Candida</i>
		<i>Teratosphaeria</i>
		<i>Ramariopsis</i>
		<i>Fimetariella</i>
		<i>Leptodontidium</i>
		<i>Sorocybe</i>
		<i>Tetracladium</i>
		<i>Knufia</i>
		<i>Cortinarius</i>
		<i>Scytalidium</i>
		<i>Marchandiobasidium</i>
		<i>Trametes</i>
		<i>Helvella</i>
		<i>Russula</i>
	<i>Trapelia</i>	
	<i>Mycosphaerella</i>	
	<i>Arnium</i>	
	<i>Loreleia</i>	
	<i>Daedaleopsis</i>	
	<i>Issatchenkia</i>	
	<i>Capronia</i>	

		<i>Cystofilobasidium</i>
		<i>Leucosporidiella</i>
		<i>Tremelloscypha</i>
		<i>Anzina</i>
		<i>Botryosphaeria</i>
	<i>Eurotiomycetes</i>	<i>Petromyces</i>
Summer	<i>Wallemiomycetes</i>	<i>Eurotium</i>
		<i>Golovinomyces</i>

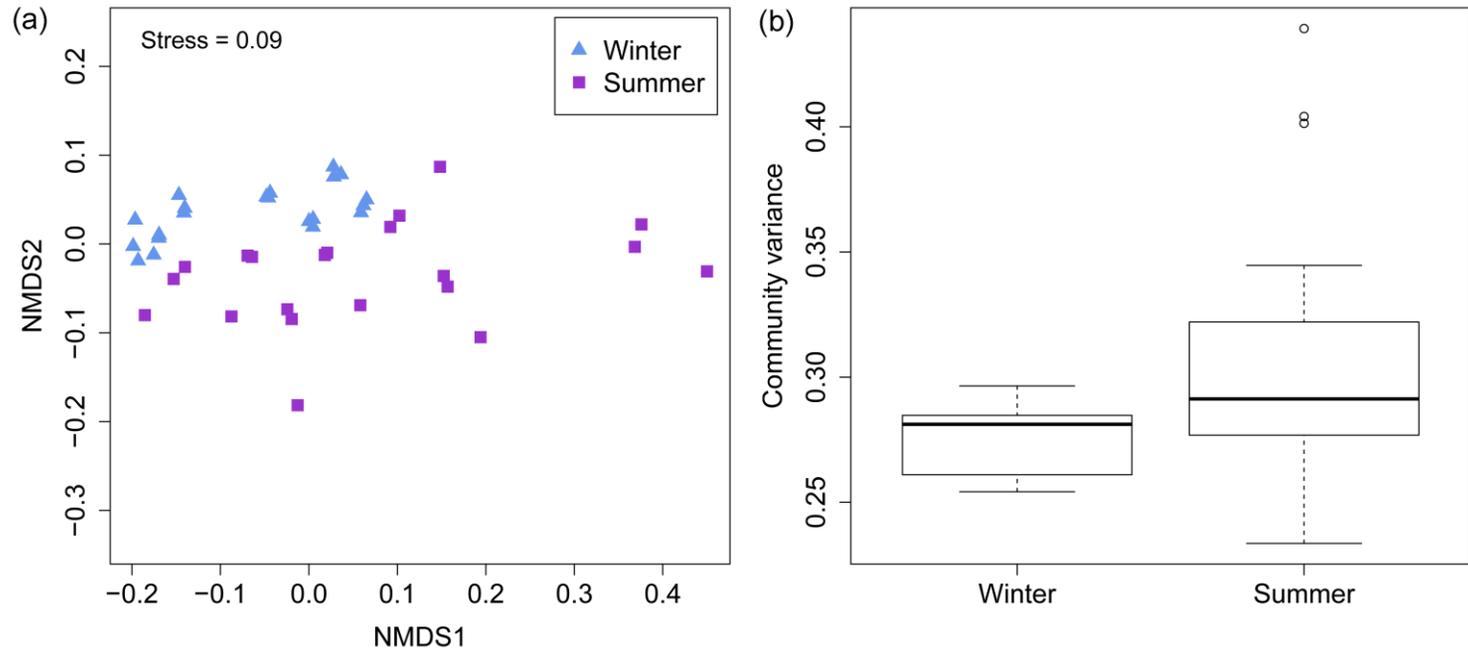


**Figure 16.** The co-occurrence network of air borne fungal communities in SCBs. Each node represents a fungal genus and lines connecting the nodes are edges.

An NMDS plot of Bray–Curtis distances showed that the composition of the airborne fungal communities was significantly influenced by seasons (ANOSIM statistic  $R = 0.96$ ,  $P < 0.01$ ; Figure 17a). Our results are consistent with the findings of several previous studies on indoor air fungi (Adams *et al.* 2013; Pitkäranta *et al.* 2008; Shelton *et al.* 2002). The beta diversity, measured as the average distance of all samples to the centroid within each season, varied significantly between seasons, with summer having significantly higher beta diversity than winter ( $P < 0.05$ ) (Figure 17b). These results suggest that the airborne fungal community composition is more heterogeneous in summer than in winter.

### **Potential human allergen/pathogen related fungal genera**

A total of 80 human allergen/pathogen related genera are known to date (Simon-Nobbe *et al.* 2008). Of these, we identified a total of 29 human allergen/pathogen related fungal genera in SCBs (Table 9). Overall, the average relative abundance of human allergen/pathogen related fungal genera was higher in winter than in summer (Figure 18). The most abundant human allergen/pathogen related fungal genus was *Fusarium* (10.8%). *Fusarium* is an emerging pathogen and can cause infections in humans, especially in immunocompromised hosts (Nucci & Anaissie 2002, 2007). Of the detected human allergen/pathogen related fungal genera, the relative abundances of four genera, namely, *Candida*, *Aspergillus*, *Pichia*, and *Trichosporon*, varied significantly between seasons, and except for *Aspergillus*, the relative abundances of the other three genera were significantly higher in winter.

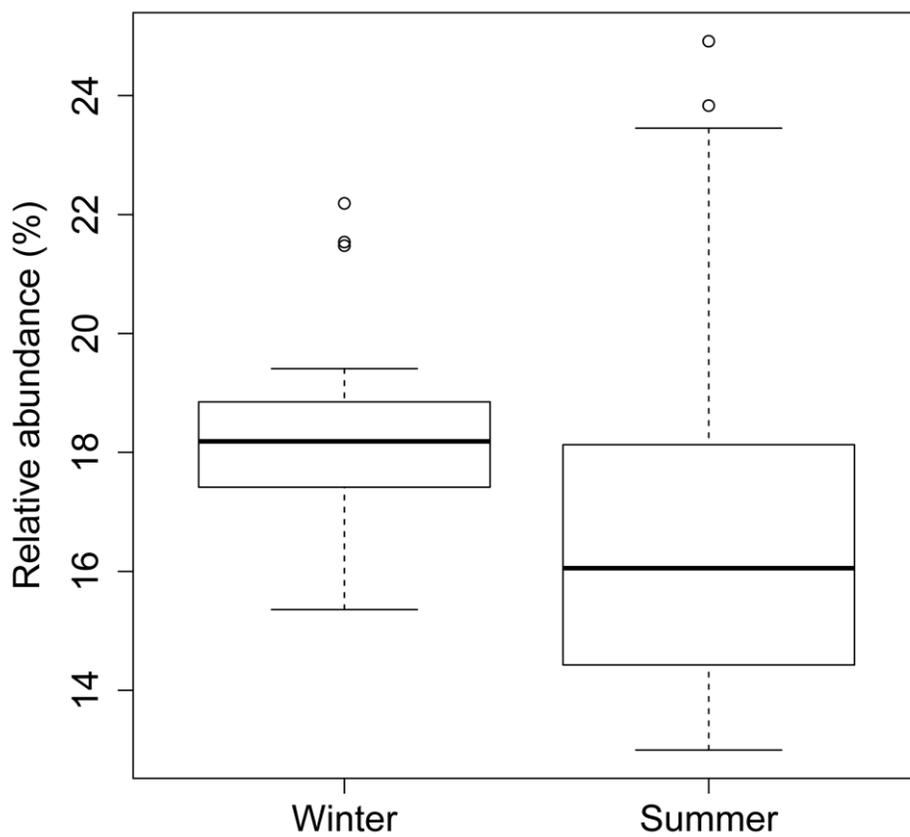


**Figure 17.** (a) NMDS plot showing fungal community composition of SCBs during the winter and summer seasons. (b) Community variance (beta diversity) of fungal community based on Bray-Curtis distance in the winter and summer seasons.

**Table 9.** Airborne fungal allergen/pathogen related genera identified in SCBs.

Name	<i>P</i> -value	Season
<i>Candida</i>	<b>0.01</b>	Winter
<i>Fusarium</i>	0.36	
<i>Rhodotorula</i>	0.22	
<i>Cryptococcus</i>	0.08	
<i>Aspergillus</i>	<b>0.01</b>	Summer
<i>Embellisia</i>	0.98	
<i>Alternaria</i>	0.11	
<i>Cladosporium</i>	0.05	
<i>Pichia</i>	<b>0.02</b>	Winter
<i>Trichosporon</i>	<b>0.01</b>	Winter
<i>Curvularia</i>	0.22	
<i>Penicillium</i>	0.90	
<i>Beauveria</i>	0.94	
<i>Ulocladium</i>	0.51	
<i>Pseudallescheria</i>	0.08	
<i>Sporothrix</i>	0.43	
<i>Chrysosporium</i>	0.11	
<i>Exophiala</i>	0.09	
<i>Stachybotrys</i>	0.22	
<i>Malassezia</i>	0.43	
<i>Epicoccum</i>	0.36	
<i>Nimbya</i>	0.98	
<i>Saccharomyces</i>	0.36	
<i>Pleospora</i>	1.00	
<i>Stemphylium</i>	0.22	
<i>Stemphylium</i>	0.22	
<i>Thermomyces</i>	0.22	
<i>Coprinus</i>	1.00	
<i>Psilocybe</i>	0.62	

All values with  $P < 0.05$  are in bold.



**Figure 18.** Box-plot showing the relative abundance of allergen/pathogen related fungal genera between winter and summer seasons in swine confinement buildings.

### **3.2.4. Conclusions**

In conclusion, through in-depth sequencing, we have shown that the airborne fungal community composition in SCBs was influenced by seasonal variations. Seasonality also influenced the alpha and beta diversities of airborne fungi in SCBs; however, both showed different patterns from one another, whereby alpha diversity peaked in winter and beta diversity peaked in summer. Several human allergen/pathogen related fungal genera were also detected in SCBs in both seasons, and in total, their relative abundance was higher in winter. These potential human allergen/pathogen related fungal genera present in the indoor air of SCBs may impact the health of farm workers, which suggests that better management practices are needed to minimize the risk of potential occupational health hazards in farm workers. However, it is to be noted that fungi are generally opportunistic pathogens, and most of the fungi detected in this study are common in the environment, so it is very unlikely that these opportunistic fungal pathogens can infect healthy individuals. Overall, this study provides a better understanding of seasonal patterns in the airborne fungal community composition and diversity in SCBs.

# **CHAPTER 4. MITIGATION OF AIBORNE CONTAMINANTS EMISSION FROM SWINE CONFINEMENT BUILDINGS**

# **Biofilter bacterial biofilm community succession reduces the emissions of airborne contaminants from swine confinement buildings**

## **4.1.1 Introduction**

The use of confinement swine buildings with high animal density has been intensified in recent years to meet the higher demand of meat products. These confinement structures emit various airborne contaminants, which can cause harmful effects to the public residing in close proximity to these buildings (detailed reviews on the airborne contaminants emitted from SCBs are mentioned in chapter 1). Therefore, reducing the emission of the airborne contaminants from SCBs is necessary in order to provide healthy environment to their neighbors.

Several mitigation strategies have been used earlier to reduce the emissions of airborne contaminants from SCBs (for detailed reviews please see the section 1.3), and it has been shown that biofilter is the most promising and cost-effective technology to reduce odorous gases emitted from livestock buildings (Estrada *et al.* 2012; Prado *et al.* 2009; Wani *et al.* 1997). Also, biofilter is an eco-friendly technology that uses no chemicals with potentially hazardous effects (Singh *et al.* 2006a; Singh *et al.* 2006b). Biofiltration is a complex process which involves interactions of absorption, adsorption, and biological degradation (Devinny *et al.* 1998). Though the performance of biofilters in reducing odorous gases were evaluated in several studies (Chen *et al.* 2008), little is known about the bacterial biofilm community immobilized in the packing material of biofilter which

helps in breaking down of the contaminants present in the air stream.

In the present study, we used a biological air filter to reduce the emissions of airborne contaminants from an experimental swine farm facility, and also investigated the successional development of bacterial biofilm community in the packing material of biofilter by using the Illumina Miseq sequencing platform. The term ‘biofilm’ being defined here as an adsorbed the thin- layered condensations of bacteria attached to the surface of cellulose pad filters. The objectives of this study were as follows:

(1) To evaluate the effect of biofilter on emission of airborne contaminants.

(2) To study the successional development of bacterial biofilm community in biofilter which helps in biological degradation of airborne contaminants present in the air stream.

## **4.1.2 Materials and methods**

### **Two-stage biofilter**

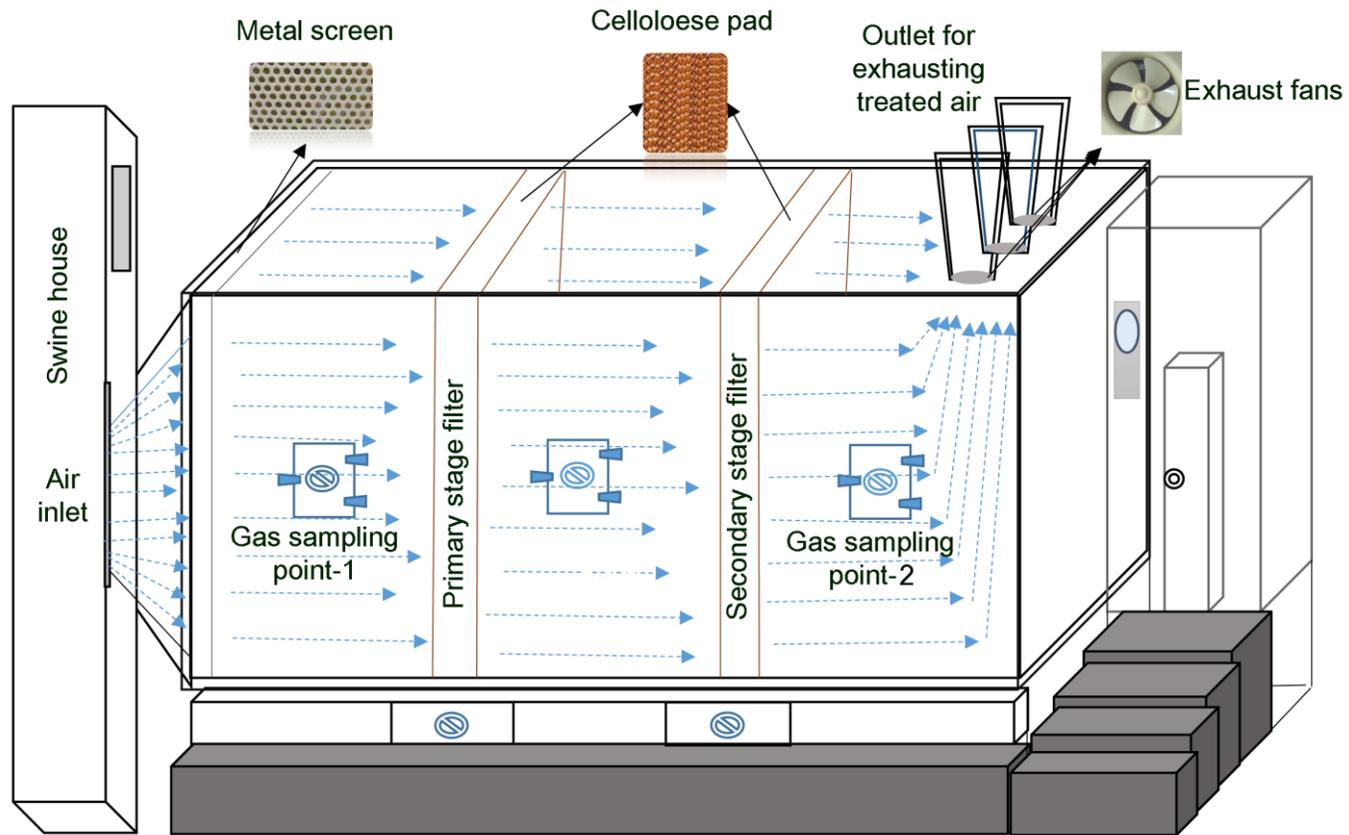
A two-stage biofilter (Figure A2) was installed next to an experimental swine farm facility (Figure A3) of Seoul National University located in Suwon, South Korea. There were 160 growing pigs were kept inside the experimental swine farm facility during the entire experiment period (11/02/2014–06/05/2014). The packing material used to design the biofilter was cellulose pads with two vertical filter walls (Figure 19). The depth of the both filters was 15 cm, and both of the filters were irrigated with recirculated water. The two-stage biofilter has designed to take up and metabolize different compounds at each stage. The first stage acts as a dust trap, where  $\text{NH}_3$ ,

dust particles and soluble organic compounds get dissolved into the water that is constantly irrigated over the filter surface. The second stage acts as a trickling biofilter, where irrigation is restricted to ensure better uptake and reduction of less soluble organic compounds by minimizing the liquid boundary layer on the surface of the biofilm. An air distribution screen was placed before first filter to ensure equal distribution of air in the biofilter and to lower the load of dust particles. The ventilation air was drawn through the biofilter from three outlets by using three ventilation fans which were placed after the biofilter.

### **Experimental setup and analysis of odorous gases**

The experiment was conducted for 12 weeks, and the reduction of odorous gases was measured every week. The odorous gases measured in this experiment were  $\text{NH}_3$ , acids-acetic acid, propionic acid, butyric acid, iso-butyric acid, valeric acid, methyl mercaptan, dimethyl sulphide, and dimethyl disulfide. Except  $\text{NH}_3$  all other odorous gases were analyzed by using gas chromatograph-mass spectrometer (Agilent GC6890N/5975C MS, Youngin, Korea) (Figure A4). For this analysis, the air samples were collected into a 1 L Tedlar bag (SKC Inc., Eighty-four, PA, USA) from the two sampling ports of the biofilter (Figure 19). After sampling, the bags were immediately transported to the laboratory and analyzed within 18 h by using solid-phase microextraction (SPME) fibers (Supelco, Bellefonte, PA, USA); the fiber type was 75-mm carboxen-polydimethylsiloxane. Samples were extracted by using SPME fibers for 30 min with a manual fibers holder from Supelco (Bellefonte, PA, USA). After extraction, the SPME fiber was removed from the Tedlar bag and immediately inserted

into the injection port of the GC-MS. The concentrations of  $\text{NH}_3$  were measure by using a GASTECH device (Pump kit No. 101).



**Figure 19.** Schematic diagram of the two-stage biofilter used in this study.

## **Sample collection and DNA extraction**

For analyzing the succession in bacterial biofilm community associated with the biofilter, samples were collected from both primary and secondary filters at days 14, 21, 35, 63, 84. The pieces of the cellulose filters were kept at 4°C until processing in the laboratory. Total DNA was extracted directly from the filters using the PowerSoil DNA isolation kit (MoBio Laboratories, Carlsbad, CA). The big pieces of the filters were aseptically cut into small pieces, loaded into the bead tube of the DNA extraction kit, and heated to 65°C for 10 min followed by 2 min of vortexing. The remaining steps of the DNA extraction were performed according to the manufacturer's instructions. The purified DNA was resuspended in 50 µl of solution S6 (MoBio Laboratories) and stored at -20°C until PCR amplification.

## **Illumina sequencing and data processing**

A single round of PCR is performed using "fusion primers" (Illumina adaptors + indices + specific regions) targeting the V6/V7/V8 regions of bacterial 16S rRNA genes (Comeau *et al.* 2011). The amplicons were sequenced at Centre for Comparative Genomics and Evolutionary Biology (CGEB), (Dalhousie University, Halifax, Canada) using paired-end (2×300 nt) Illumina sequencing with a MiSeq system (Illumina, USA). The mothur software package was used to process the sequence data (Schloss *et al.* 2009). First, paired-end sequence assembly was generated using the '*make.contigs*' command in mothur prior to quality trimming, sequence filtration and alignment against a SILVA alignment (<http://www.arb-silva.de/>). Next, the '*pre.cluster*' and '*chimera.uchime*' commands in mothur were used to

remove the sequencing errors and chimeric sequences, respectively (Edgar *et al.* 2011; Huse *et al.* 2010). Taxonomic annotations of all of the high quality sequences were obtained via '*classify.seq*' command in mothur using the reference Greengenes taxonomy database. A random subset of 17,852 sequences per sample was generated using the '*sub.sample*' command in mothur prior to statistical analysis. The bacterial operational taxonomic unit (OTU) matrix was built using '*dist.seqs*' command in mothur, and the generated distance matrix was used to cluster sequences into OTUs by mothur's '*cluster*' command using the average linkage algorithm. Finally, '*make.shared*' command was used to generate the bacterial OTUs at a cutoff value of 0.03, and the entire singleton OTUs were removed prior to analysis. The diversity indices were calculated using '*summary.single*' command in mothur.

### **Statistical processing and analysis of results**

The reduction efficiency of each odorous gas was determined using the relationships between the influent and effluent gas phase concentration, as follows:

$$\text{Reduction efficiency (\%)} = [(C_{\text{in}} - C_{\text{out}}) / C_{\text{in}}] \times 100$$

Where,

$C_{\text{in}}$  = Influent gas phase concentration

$C_{\text{out}}$  = Effluent gas phase concentration

The odorants reduction efficiencies of biofilter were correlated

to the sampling time using linear functions. The differences in bacterial community composition based on Bray-Curtis distances were visualized using non-metric multidimensional scaling (NMDS) plots. A permutational multivariate analysis of variance (PERMANOVA) was performed with 999 permutations using the Adonis function in VEGAN R package to test if bacterial biofilm communities of biofilter differed significantly by filtration stage and sampling time.

### **4.1.3 Results and discussion**

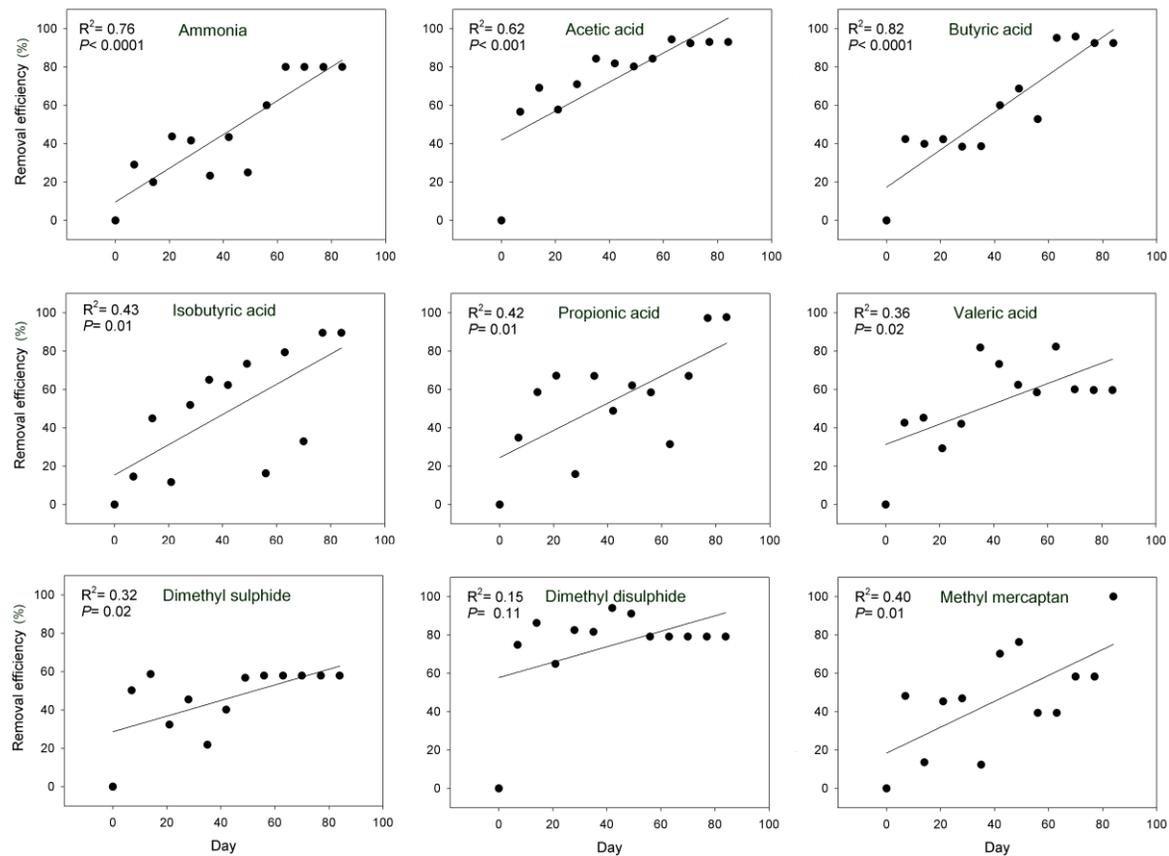
#### **Odor reduction efficiency of biofilter**

A total of nine odorous gases were measured during this experiment and the results indicate that all these odorants emissions were efficiency reduced by biofilter in efflux air from SCBs (Figure 20). The odorant reduction efficiency of biofilter was increased linearly with time (Figure 20). The reduction efficiency of methyl mercaptan was highest (100%) at day 84, whereas the reduction efficiency of dimethyl sulphide was lowest (57.9%) at day 84. These results are in agreement with other studies where biofiltration system was used to reduce the emissions of gaseous compounds from SCBs, and it has been shown that biofilter allows a 64–69% decrease in  $\text{NH}_3$  and an 85–92.5% reduction in other odorous gaseous compounds (Sheridan *et al.* 2002).

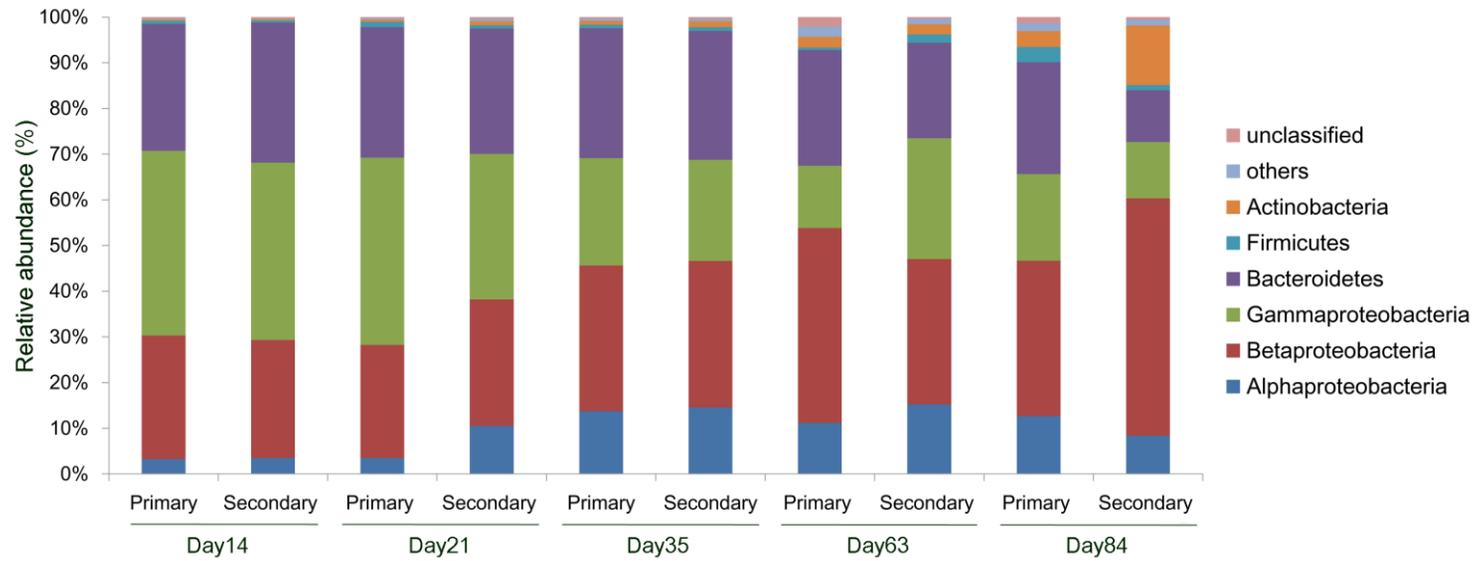
#### **Successional development of bacterial biofilm community**

The most abundant bacterial phyla across all samples were *Proteobacteria* (66.5%), followed by *Bacteroidetes* (22.9%) and to a lesser degree, *Firmicutes* (6.1%), and *Actinobacteria* (3.5%), while 0.6%

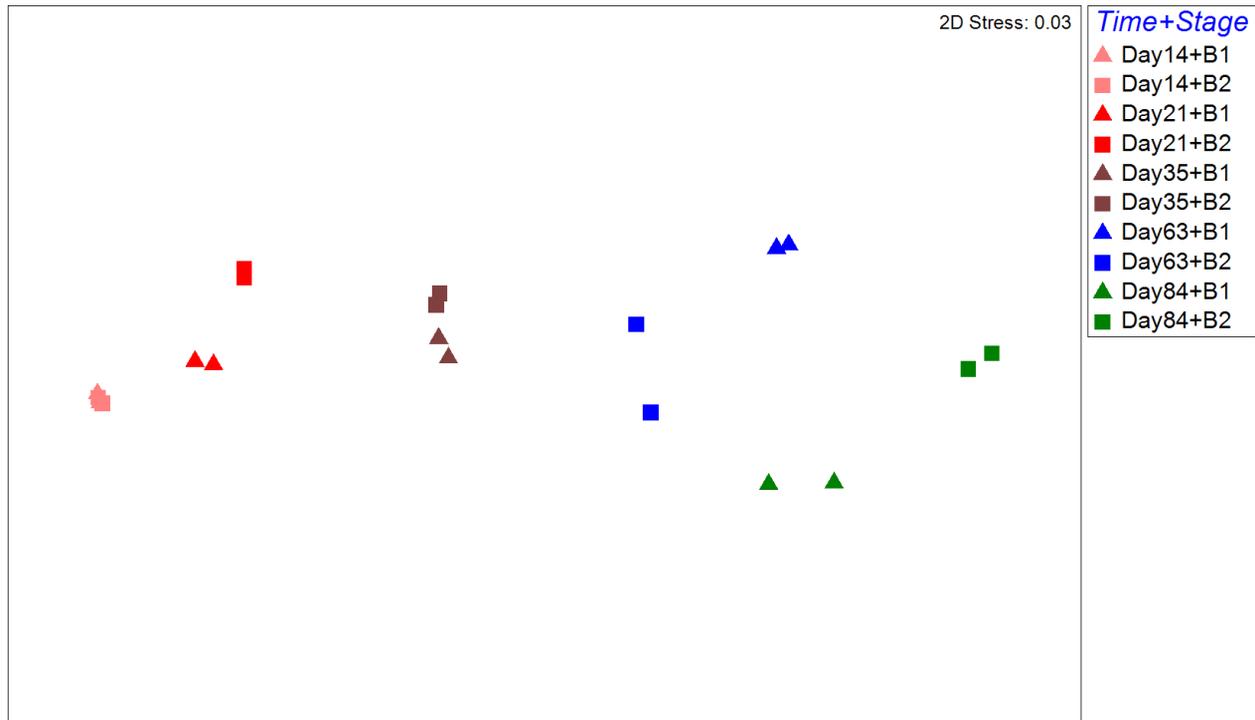
of the sequences were unclassified (Figure 21). Similar community compositions have been observed from a full-scale biofilter treating swine house exhaust air (Kristiansen *et al.* 2011b), and also from other air filter biofilms (Borin *et al.* 2006; Friedrich *et al.* 2002; Friedrich *et al.* 2003). These results indicate that the bacterial biofilm community of biofilter was specialized and adapted to the unique environmental conditions. A non-metric multidimensional scaling (NMDS) using Bray-Curtis distance showed that bacterial biofilm community on biofilter was strongly influenced by time (Figure 22). A permutational multivariate analysis of variance (PERMANOVA) results showed that bacterial biofilm community composition of biofilter was strongly influenced by time ( $F = 52.6$ ,  $P < 0.0001$ ; Table 10) which explained 84% of the variation in community composition. The filtration stage ( $F = 5.1$ ,  $P = 0.02$ ; Table 10) and the interaction between time and filtration stage ( $F = 6.2$ ,  $P = 0.0003$ ; Table 10) also significantly influenced the bacterial community composition, however, the explained proportion of the variations were lower compared to time (filtration stage = 2%, time x filtration stage = 11%). The successional change in bacterial biofilm community of biofilter with time was reported earlier (Portune *et al.* 2015), and it has been also observed that bacterial community structure changes along the filtration stages of the biofilter (Kristiansen *et al.* 2011b).



**Figure 20.** Relationship between time and biofilter reduction efficiency of various odorous compounds.



**Figure 21.** Relative abundances of dominant bacterial taxa across time point and filter stage.



**Figure 22.** NMDS plot of Bray–Curtis dissimilarities between samples at different time point and two stages

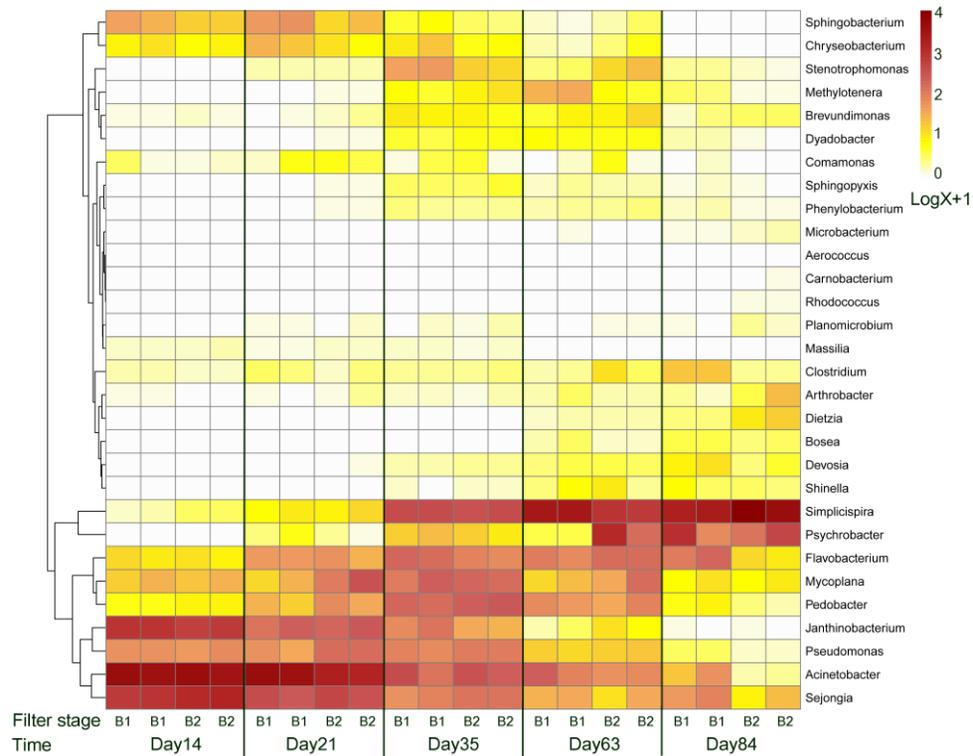
**Table 10.** Results of multivariate PERMANOVA verify significant differences in bacterial community composition between treatment groups.

	Bacterial community composition				
	d.f	MS	F. Model	R <sup>2</sup>	<i>P</i> value
Time	4	0.74	52.62	0.84	0.0001
Filter stage	1	0.07	5.07	0.02	0.02
Time x Filter stage	4	0.09	6.23	0.11	0.0003
Residuals	10	0.01		0.04	
Total	19			1.00	

During the initial time point of the experiment (day 14) *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria* and *Bacteroidetes* were the most abundant bacterial phyla across both primary and secondary stage filters (Figure 21). At the mid time point of the experiment (day 35) the relative abundance of *Gammaproteobacteria* decreased with increase in the relative abundance of *Alphaproteobacteria* and *Betaproteobacteria*, however, the relative abundance of *Bacteroidetes* remained similar (Figure 21). At the late time point (day 84), the relative abundance of *Betaproteobacteria* increased further compared to mid time point and the relative abundance of *Gammaproteobacteria* and *Bacteroidetes* were decreased, however, the relative abundance of *Alphaproteobacteria* did not show much variation at late time point compared to mid time point (Figure 21). The members of the *Proteobacteria* and *Bacteroidetes* are reported to degrade various odorants (Ralebitso-Senior *et al.* 2012), and dominance of different subgroups of the bacteria phyla at the later time of the experiment made biofilter more efficient in reduction of a wide variety of odorants. Interestingly, the relative abundance of the phylum *Actinobacteria* increased sharply at late time point of the experiment (Figure 21). The members of *Actinobacteria* are known to degrade butyric acid and dimethyl disulfide (Kristiansen *et al.* 2011a).

The comparison of relative abundance of bacterial biofilm community at the genus level also revealed many apparent relationships to time (Figure 23). The heat map of 30 most abundant bacterial genera showed that among the dominant genera in these samples, no single genus was abundant at all time point, although each shows its own

pattern peaking at early, mid or late time points (Figure 23). Most of the abundant genera found in this study are known to degrade various odorants (Table 11).



**Figure 23.** Heat map showing the relative abundance ( $\log x + 1$  transformed) of 30 most dominant bacterial genera at each time point and filter stage (B1=Primary stage and B2=Secondary stage).

**Table 11.** The known odorant degrading bacterial genera found in this study.

<b>Genera</b>	<b>Odorant/class</b>	<b>References</b>
<i>Acinetobacter</i>	Hydrogen sulfide	(Omri <i>et al.</i> 2011)
<i>Arthrobacter</i>	Hydrogen sulfide, ammonia, methylamine, dimethylamine and trimethylamine	(Chung <i>et al.</i> 2001; Ho <i>et al.</i> 2008)
<i>Clostridium</i>	Nitric oxide	(Chen <i>et al.</i> 2009)
<i>Devosia</i>	VOC	(Friedrich <i>et al.</i> 2002)
<i>Dietzia</i>	Dimethyl sulfide and butyric acid	(Kristiansen <i>et al.</i> 2011a)
<i>Flavobacterium</i>	Butyric acid, dimethyl disulfide and other VOC	(Friedrich <i>et al.</i> 2002; Kristiansen <i>et al.</i> 2011a)
<i>Microbacterium</i>	Dimethyl sulfide, butyric acid and dimethyl disulfide	(Kristiansen <i>et al.</i> 2011a; Shu & Chen 2009)
<i>Pedobacter</i>	VOC	(Friedrich <i>et al.</i> 2002)
<i>Pseudomonas</i>	Hydrogen sulfide, dimethyl sulfide, ammonia and VOC	(Omri <i>et al.</i> 2011; Shu & Chen 2009)
<i>Psychrobacter</i>	Ammonia, hydrogen sulfide, dimethylamine, trimethylamine, isobutyric acid	(Gutarowska <i>et al.</i> 2014)
<i>Rhodococcus</i>	Dimethyl sulfide, butyric acid, ethyl acetate, o-xylene, and VOC	(Aldric & Thonart 2008; Jeong <i>et al.</i> 2008; Kristiansen <i>et al.</i> 2011a)
<i>Simplicispira</i>	Dimethylamine	(Liao <i>et al.</i> 2015)
<i>Sphingobacterium</i>	Butyric acid, dimethyl disulfide, dimethylamine and VOC	(Friedrich <i>et al.</i> 2002; Kristiansen <i>et al.</i> 2011a; Liao <i>et al.</i> 2015)
<i>Stenotrophomonas</i>	Ammonia and VOC	(Friedrich <i>et al.</i> 2002)

#### **4.1.4 Conclusions**

In conclusion, biofilter system used in this study was found to reduce various odorants emission efficiently from swine farm facility. Bacterial biofilm community structure of biofilter exhibited a strong time successional pattern, and to a lesser extent, filtration stage and the interaction between time and filtration stage also lead to a significant variation in community structure of bacterial biofilm. Certain bacterial phyla and genera with ability to degrade various odorants got enriched at later time point of the experiment which might result in reduction of a wide variety of odorants. Analysis of the bacterial biofilm community of biofilter system in the present study represents an important first step toward understanding the stability and efficiency of such biofilters.

## GENERAL CONCLUSIONS

The high animal densities in SCBs lead to poor indoor air quality. The airborne contaminants present in SCBs affect both animal and human health. Although, the indoor airborne contaminants have been analyzed in several studies in SCBs, relatively little is known about the factors influencing the abundance and community composition of bioaerosols in SCBs. Also, despite growing awareness of health risk in the neighboring resident community, the mitigation strategies of airborne contaminants emission from SCBs are poorly studied. This study investigated the indoor bioaerosols community structure and diversity in SCBs, and how the bioaerosol communities are affected by manure removal system and seasonal variations. In this study, a biofiltration system was also used to study how and to which extent it helps in mitigation of airborne contaminants emissions from SCBs.

At first, the effect of three different types of manure removal systems (deep-pit manure removal with slats, scraper removal system, and deep-litter bed system) was investigated on the abundance and composition of airborne biotic contaminants of SCBs. The manure removal system was found to strongly influence the abundance and community composition of airborne biotic contaminants of SCBs. The airborne biotic contaminants were most abundant in SCBs equipped with a deep pit with slats. *Firmicutes* and *Actinomycetes* appear to be the dominant bacterial phyla in SCBs, and a comparison of relative abundances of these phyla together with dominant genera strongly supports the concept that individual bacterial lineages found in SCBs

are enriched to specific manure removal systems. The present study represents a first glimpse of the airborne biotic contaminants of SCBs with different types of manure removal systems using next-generation sequencing methods.

The next objective of this study was to investigate the seasonal variations in community composition and the diversity of airborne contaminants (bacteria, fungi and tetracycline resistance genes) in SCBs. The results indicate that seasons have an influence on the biotic contaminants abundance, community composition and diversity, in indoor air of SCBs. Seasonality was significantly associated with microclimate variables, indicating that indoor environmental conditions play an important role in structuring airborne biotic contaminants in SCBs. Several human allergen/pathogen related fungal genera were also detected in SCBs in both seasons, and in total, their relative abundance was higher in winter. These potential human allergen/pathogen related fungal genera present in the indoor air of SCBs may impact the health of farm workers, which suggests that better management practices are needed to minimize the risk of potential occupational health hazards in farm workers. However, it is to be noted that fungi are generally opportunistic pathogens, and it is very unlikely that these opportunistic fungal pathogens can infect healthy individuals.

In the final objective of this study, a biological air filter system was used to reduce the emissions of airborne contaminants from SCBs, and also investigated the successional development of bacterial biofilm community in the packing material of biofilter by using the Illumina Miseq sequencing platform. In this study, it has been observed that the

odorant reduction efficiency of biofilter was increased linearly with time. The results also indicate that bacterial biofilm community structure of biofilter exhibited a strong time successional pattern, and to a lesser extent, filtration stage and the interaction between time and filtration stage also lead to a significant variation in community structure of bacterial biofilm. Certain bacterial phyla and genera with ability to degrade various odorants got enriched at later time point of the experiment which might result in reduction of a wide variety of odorants.

Overall, it appears that patterns of community composition and diversity of bioaerosols in SCBs were strongly influenced by manure removal system and seasonal variations. It has been also observed in this study that biofilter could be used as efficient and cost effective option to reduce the emissions of airborne contaminants from SCBs. Together these results provide a baseline framework with which better management practices and regulations can be designed to minimize the potential health impact on both the farm workers and the public residing in close proximity to these buildings.

### **Significance of this study for swine farmers**

The following are the significance of this study for swine farmers:

- 1) In this study, it has been found that the manure removal system strongly influence the airborne biotic contaminants present in swine confinement buildings. Adoption of better management practices, such as proper cleaning of swine manure and optimizing the hosing conditions could minimize these airborne contaminants in SCBs.

- 2) Seasonal trend in airborne biotic contaminants is also recognized in this study, with winter season peaked in the abundance and diversity of airborne contaminants due to minimal ventilation rate to prevent the heat loss during winter. The abundance, composition, and diversity of these contaminants were found significantly associated with microclimatic parameters of SCBs, particularly air speed, PM<sub>2.5</sub> and TSP, which suggests that by controlling the microclimate parameters the concentrations of airborne biotic contaminants can be reduced to minimize potential health impacts on both livestock and humans working in SCBs.
  
- 3) Odorous gas emissions from SCBs have been of increasing concern in the South Korea. In this study, a biofilter system has been successfully used to reduce odorous gas emissions from an experimental swine farm facility. This biofilter system enriched the odor degrading native airborne microbial community of swine house. This biofilter system could be used as an efficient and cost effective option to reduce the emissions of odorous gases from SCBs.

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## APPENDIX



**Figure A1.** A Gilian air sampler (Sensidyne Inc., Clearwater, FL, USA) used in this study for collecting aerosol samples from SCBs.



**Figure A2.** (a) Outdoor and (b) indoor view of swine farm facility of Seoul National University at Suwon, South Korea.



**Figure A3.** A two-stage biofilter installed next to an experimental swine farm facility of Seoul National University at Suwon, South Korea.



**Figure A4.** Gas chromatograph-mass spectrometer (Agilent GC6890N/5975C MS, Youngin, Korea) used in this study to determine the concentration of odorous gases.

## ABSTARCT IN KOREAN

무창돈사 (SCB) 내 부유세균의 풍부도 및 군집구성 그리고 그 감소에 영향을 미치는 주요 요인에 대한 연구는 거의 이루어진 적이 없다. 본 연구에서는 돈사 내에서 실내 부유세균 군집 구조와 다양성에 대한 연구를 차세대 염기서열 분석 기술을 이용하여 수행하였다. 이 차세대 염기서열 분석법을 이용하여 유형별 돈분뇨 제거시스템 및 계절적 변화에 따른 부유세균 군집에 대한 영향의 영향을 조사하였다. 또한 본 연구에서는 생물학적 여과시스템을 사용하여 이것이 돈사로부터 배출되는 공기오염 물질의 완화에 얼마나 도움이 되는지 및 그 방법에 대하여 연구하였다.

Cultivation-independent 방법을 이용한 분뇨제거 시스템 유형 (슬랫-피트 분뇨제거시스템, 스크레이퍼 분뇨제거시스템, 깔개시스템)이 돈사 내 부유생물 오염물질의 풍부도 및 조성에 미치는 영향을 연구하였다. 16S rRNA 및 유전자 여섯 테트라사이클린 내성 유전자 (tetB, tetH, tetZ, tetO, tetQ 및 tetW) 의 풍부도 존재비는 실시간 PCR 을 사용하여 정량화하였다. 16S rRNA 유전자 및 tetB 유전자를 제외한 테트라사이클린 내성 유전자의 풍부도는 슬랫-피트 돈사에서 유의하게 높았다. 이러한 결과는 기존의 배양 기반 연구의 결과와 대조된다. pairwise Bray-Curtis distances 방법으로 측정된 부유세균의 군집조성은 분뇨제거시스템 유형에 따라 유의한 변화를 보였다. 16S rRNA 기반 pyrosequencing 결과, *Firmicutes* (72.4%)가 우점군으로 *Lactobacillus* 와 함께 주요 종으로 나타났고, 반면 *Actinobacteria* 는 검출세균의 10.7 %로 나타났다. *Firmicutes* 는 칸막이가 있는 슬랫-피트 분뇨제거시스템 돈사 내에 더 많이 분포하였고, 반면 *Actinobacteria* 는 깔개돈사 내에 매우 풍부하게 분포하였다. 전반적으로, 이 연구의 결과는 무창돈사의 가축분뇨처리시스템 유형이 내 공기 중 부유세균 의 풍부도와 구성을 구조화하는데 중요한 역할을 함을 제시하였다.

무창돈사 내 부유세균의 계절적 역학에 대해서는 거의 알려져 있지 않다. 본 연구에서는 7개 농장의 돈사 내 부유세균을 여름과 겨울에 한 차례 씩 방문하여 관찰하였다. 16S rRNA 유전자, V3 영역에 대한 paired-end Illumina 염기서열 분석방법으로 세균 군집 구성과 다양성의 계절적 변화를 조사하였다. 16S rRNA의 유전자와 여섯 개의 테트라사이클린 내성 유전자(tetB, tetH, tetZ, tetO, tetQ 및 tetW)의 풍부도를 실시간 PCR을 이용하여 정량화하였다. 박테리아 풍부도, 군집구성 및 다양성은 겨울에 최대로 분석되어 강한 계절적 양상을 보였다. 무창돈사 내 미기상 변수 특히 유속, PM2.5 및 총부유 입자(TSP)가 세균의 풍부도 및 군집구성, 부유세균의 다양성에 유의한 상관관계가 있는 것으로 나타났다. 계절적 변화도 네 가지 테트라사이클린 내성 유전자, tetH, tetO, tetQ 및 tetW에서 관찰되었다. 이러한 내성 유전자의 발생빈도는 겨울 동안 수집된 표본에서 유의하게 높았으며, 또한 유속, PM2.5 및 TSP와 유의한 상관관계가 관찰되었다. ~~있었다.~~ 결론적으로, 이러한 결과는 무창돈사 내 부유세균 계절적 동향을 보이는 것으로 분석되며, 이들은 무창돈사 미기상 변수들과 상관 있음을 알 수 있었다.

무창돈사 내에서 계절의 변화가 부유 곰팡이의 군집 조성 및 다양성에 미치는 영향에 대해서도 연구하였다. 부유물 표본은 겨울 및 여름에 일곱 개의 양돈장에서 수집하였다. 리보솜 유전자의 내의 ITS region 1은 paired-end Illumina 염기서열 분석방법으로 서열화하였다. 세균과 마찬가지로, 실내 부유곰팡이의 군집구성 및 다양성은 계절변화에 의해 영향을 받음을 관찰하였다. 그러나, 알파와 베타 다양성은 서로 매우 다른 양태를 보였는데, 알파 다양성은 겨울에 정점을, 베타 다양성은 여름에 정점을 나타내었다. 여러 인체 알레르기 항원/병원체 관련 곰팡이 종(種)이 무창돈사 내에서 확인되었다. 이러한 인체 알레르기 항원/병원체 관련 곰팡이 종 중, *Candida*, *Aspergillus*, *Pichia*, *Trichosporon*은 계절에 따라 크게 변화하였다. 일반적으로, 인체 알레르기

항원/병원체 관련 곰팡이 종의 상대빈도는 여름보다 겨울에 더 높았다.

무창돈사로부터 배기(排氣)의 오염물질을 희석시키는 데는 바이오필터시스템이 경제적인 설비로 알려져 있다. 그러나, 공기흐름 내 존재하는 오염 물질의 분해에 도움이 되는 바이오 필터 표면에 고정된 세균 바이오 필름내의 군집구조에 대해서는 알려져 있지 않다. 생물학적 공기 여과시스템은 양돈사 내 공기 중 오염물질의 배출을 감소시키기 위해 사용되며, 또한 Illumina Miseq 염기 서열 분석기술을 이용하여 바이오 필터의 포장재 내 세균 바이오 필터의 군집의 발달과정에 대하여 조사하였다. 그 결과 바이오 필터의 악취감소 (냄새제거) 효율이 시간에 따라 선형적으로 증가함을 관찰하였다. 연구결과에 따르면 바이오 필터의 박테리아 군집에 존재하는 바이오 필터 구조 또한 강한 시간 연속적인 양상을 보이고, 보다 적게는, 여과 단계와 시간 및 여과 단계 사이의 상호작용 또한 세균 바이오필터의 군집 내에서 매우 다양하게 나타났다. 각종 악취물질을 분해하는 능력을 지닌 특정 세균 문(門) 및 종(種)이 실험 후기 시점에 농축되어 다양한 종류의 악취가 감소됨을 관찰하였다.

결론적으로, 실내 부유세균의 군집 구성 및 다양성은 분뇨제거 시스템 및 계절변동에 따라 크게 달라지는 것으로 밝혀졌다. 또한 바이오필터시스템이 효율적으로 무창돈사에서 발산되는 다량의 악취기체의 배출을 감소시키는 것이 관찰되었고, 세균 바이오필름의 군집의 천이 및 악취 제거의 상관관계는 축사내 작업자와 축사주위 정주민에 대한 잠재적 건강위해적 영향을 최소화하기 위한 더 나은 관리 전략 수립에 도움이 될 수 있을 것이다.

**키워드:** 부유세균, 바이오필터, 세균, 곰팡이, 일루미나, ITS, 가축분뇨처리시스템, pyrosequencing, 계절변화, 무창돈사, 16S rRNA 유전자.

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