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Faeces As an Important Pollution Source of Airborne Pathogens in Traveling from Swine Farm

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Abstract: Airborne pathogens are the most important factor causing environmental issues on pig farms. Research on airborne swine-derived microbes has mainly concentrated on several specific microbes or pathogens. The present work was conducted to detect and identify the entire microbial community in piggery air and their dispersion by 16SrRNA sequencing. Fifteen faeces and eighty-four air samples were collected in swine barns and from different distances away from the barns, respectively. The results showed that the faeces and air share the most dominant bacteria. The top 10 genera belonged to Fimicutes, Proteobacteria and Bacteroidetes, and accounted for 54% and 76-84% of the total sequences. Moreover, great higher ($P < 0.01$) microbial diversity was detected in the faeces. This study indicated that a hygienic interval of 50 m should be set on swine farms to prevent the spreading infectious disease caused by airborne pathogens.

Keywords: air microorganism, 16SrRNA high-throughput sequencing, manure, piggery, swine

1. Introduction

The air inside and downwind of swine barns can contain high amounts of air pollutants, including gases, dust, fungi and bacteria classified as zoonotic agents, endotoxins and allergens (Malmberg et al. 1990, Dutkiewicz et al. 1994), which are also considered to be bioaerosols (Dungan 2012). These bioaerosols can present health risks for the animals in confined buildings, as well as for humans working in this atmosphere (Madelin & Wathes 1989, Whyte 1993, Bull et al. 2006, Hartung & Schulz 2008). Recently, concern has risen that these bioaerosols, when emitted from the animal houses through the exhaust air, may also pose a health risk for nearby residents or animals in neighbouring farms (Millner 2009). Some earlier experiments measured viable bacteria emitted from swine barns and found that *E.coli* was detected as far as 100 m away (Duan et al. 2009).

In the previous studies, only a small portion of the bacterial community was investigated by using older methods such as fluorescence in situ hybridization (FISH), denaturing gel gradient electrophoresis (DGGE) and terminal restriction fragment length polymorphism (T-RFLP) (Osborn et al. 2000, Pernthaler et al. 2002). The high-throughput sequencing method has been widely used for measuring all bacteria in swine environments, such as air and wastewater (Vestergaard et al. 2018, Yang et al. 2021). As an essential carrier of microorganisms, swine faeces has also been investigated in the microbial community for further treatment (Nakamura et al. 2020). However, little research focused on pathogens' transmission from faeces to air. The travel distance of airborne pathogens and their bacterial community composition is unclear.

In the present study, the 16Sr RNA high-throughput sequencing method was used to identify all airborne and faeces bacteria in the same farm to investigate the (1) diversity and composition differences; (2) sharing bacteria between faeces and air; (3) dispersion of pathogens in both faeces and air. The results can guide the biosecurity management of swine farms.

2. Materials and Methods

2.1. The swine farm description

The sampling was performed on a swine farm in the summer. The farm is located in the Sichuan basin in China, which is characteristically hilly with many large trees. The elevation is about 600 m above sea level. The wind direction around the farm location is irregular, and the main wind direction is not apparent. There were five kinds of a barn on the farm: gilt barn, mating barn, gestation barn, growing barn and finishing barn. A slatted floor was used for manure management. The workers provided feed, and water was supplied by automatic drinking nozzles. Vaccines were applied for swine routinely. Also, the researchers and workers used strict hygiene to prevent cross-infection.

2.2. The faeces and air samples collection

The faeces samples were collected in swine barns using sterilized tweezers with 5 ml sterilized phosphate-buffered saline (PBS, Beijing Aoboxing Bio-tech Co., Ltd., Beijing, China). Fifteen manure samples weighing about 2 grams were collected on the floor of five kinds of barns. In each barn, three sampling points were located at the midpoint and both ends of the diagonal. The faeces samples in centrifuge tubes were put into liquid nitrogen containers and returned to the lab on the collection day. The collection was carried out in different sites in the barn and ranged from 9:30 to 21:30.

A glass impingement airborne microorganism sampler (SKC BioSampler, SKC Inc., USA) was applied for bioaerosol collection as described by Kembel et al. (2012). The separation of the particulates from the air stream occurs by collision with a liquid surface in an impinger. All samplers were pasteurized in the lab at 121°C for 30 min in advance. Sterile phosphate-buffered saline (PBS) (20-mL) was added into the collection vessel as a medium, and the liquids were maintained sterile before use. Eighty-four samples were collected at 0, 10, 20, 50, 100, 200 and 400 m away from the farm wall at the height of 1.5 m: 12 samples at each distance. Based on the wind direction before sampling, the sample collection sites were upwind (6 samples) and downwind (6 samples) from the farm. The 12 samples from 0 m were collected at the centre of the gilt barn, the mating barn, the gestation barn, the growing barn and the finishing barn. The collection time ranged from 09:30 to 21:30. Aerosol samples were collected by drawing the gas at a flow rate of 12.5 L/min for 30 min into the PBS collection medium. As previously described, the samplers were flash-frozen in liquid nitrogen in centrifuge tubes and returned to the lab on the collection day.

During bioaerosol sampling, the indoor and outdoor temperature (T), relative humidity (RH) and air flow rates were recorded at the exact location. Temperature and RH were measured with a Dewpoint Thermohygrometer (WD-35612, OAKTON, Germany). Air flow rates were measured by a portable anemometer (HD2303, Delta OHM, Italy). Climatic results are presented in Table 1. The indoor and outdoor T, RH and air flow rates were 26-31°C and 25-34°C, 92-99% and 77-99%, 0.09-0.65 m/s and 0.01-0.91 m/s, respectively.

Table 1. Climatic parameters of swine farm during sampling

Sampling sites	T (°C)	RH (%)	Air flow rate (m/s)
Inside barn	25.6-30.7	91.5-98.5	0.09-0.65
Outside barn	25.4-34.2	77.3-99.1	0.01-0.91

Note: The air was sampled at 9:30-21:30 in July and August, 12 samples in each group.

2.3. DNA extraction and sequencing

Total DNA was extracted from faeces and air samples using the E.Z.N.A.® Stool DNA Kit (TaKaRa, Japan) and the E.Z.N.A.® Water DNA Kit (TaKaRa, Japan). Universal primers (F16S-27/R16S-1492) were used to amplify the segment of eubacterial 16S rRNA of air samples (Martin-Laurent et al. 2001). DNA was quantified using a Qubit 3.0 Fluorometer (Life Technologies, Shanghai, China). 16S rRNA gene amplicons were produced and submitted for sequencing on V4 (515F-806R) gene fragments. The PCR primers used were previously described by Zeng et al. (2017) (515F: 5′-GTG CCA GCM GCC GCG GTA A-3′ and 806R: 5′-GGA CTA CHV GGG TWT CTA AT-3′). The PCR amplification reaction was performed at a final volume of 50 μL and followed by 30 cycles of 95°C for 7 min, 94°C for 1 min, 55°C for 1 min, and 68°C for 8 min. Both the PCR product from air samples and the extracted DNA from faeces samples were identified by 1% wt/vol Agarose-Gel Electrophoresis. The obtained 15 amplicons from each faeces sample were mixed to be three group samples, while the 12 air samples from each distance were combined to be three group samples for sequencing, which was performed on an Illumina HiSeq 2×250 (Illumina Ltd., Diego, California, USA).

2.4. Analyses of sequencing data

Sequence quality control and analyses were performed using the QIIME pipeline (Caporaso et al. 2010). Sequences were first quality filtered following previously published recommendations and then screened for chimaeras using the Usearch7.0 software and the Silva database (silva.gold.ng.fasta), filtered and then picked using UCLUST (Koubová et al. 2012). The operational taxonomic units (OTUs) picking was done under a similarity of over 97% using the database on the Greengenes website (http://greengenes.secondhenome.com/). Any OTUs clustered with less than 10 reads were manually removed to ensure that unique OTUs were not over-estimated. Mothur (V1.32) and R software (Zeng et al. 2017) were used for further analysis.

2.5. Statistical methods

All data were analyzed by one-way analysis of variance (ANOVA) using SPSS version 27.0 software at a 95% confidence level to assess the group differences.

3. Results and Discussion

3.1. Bacterial community structure in both air and faeces

A total of 20421 sequences without chimaeras and 6855 sequences with chimaeras were obtained after processing 21 DNA samples (3 faeces samples and 18 air samples). The taxon was performed to reveal the community profile of the bacteria in faeces and air from the swine farm. There were 27, 70, 130, 237 and 504 identified bacteria at the Phylum, Class, Order, Family and Genus levels, respectively.

The relative abundance of bacteria at the phylum level was displayed in Fig. 1A. The top 3 phyla Fimicutes, Proteobacteria and Bacteroidetes accounted for about 96-98% of the total bacterial gene sequences, which was following the previous reports (Song et al. 2021). The Fimicutes was the predominant phylum both in faeces and air, followed by Proteobacteria were more abundant in the air, and Bacteroidetes were more abundant in faeces. A significant difference ($P < 0.05$) was found between faeces and air.

Moreover, the top 10 bacteria in faeces (54%) and air (76-84%) belonged to Fimicutes, Proteobacteria and Bacteroidetes are presented in Fig. 1B. Genera *Prevotella*, *Pseudomonas,* and unclassified Ruminococcaceae were the dominant bacteria in faeces, whereas they were Lactococcus, Pseudomonas, Acinetobacter in air. Significant relative abundant difference ($P < 0.05$) was recorded between faeces and air. Specifically, the abundance of Prevotella, unclassified Ruminococcaceae and unclassified Clostridiales in faeces (17%, 14% and 4%, respectively) were significantly higher than in air (0-0.05%, 0.35-0.60% and 1.13-1.80% in air at different distances). On the contrary, the relative abundance of *Lactococcus*, *Pseudomonas*, *Acinetobacter*, *Flavobacterium*, *Streptococcus* and unclassified Enterobacteriaceae in the air was higher than in faeces and *Chryseobacterium* in faeces was under the detection limit. Similar top 10 genera in swine manure were reported by Hao et al. (2021).

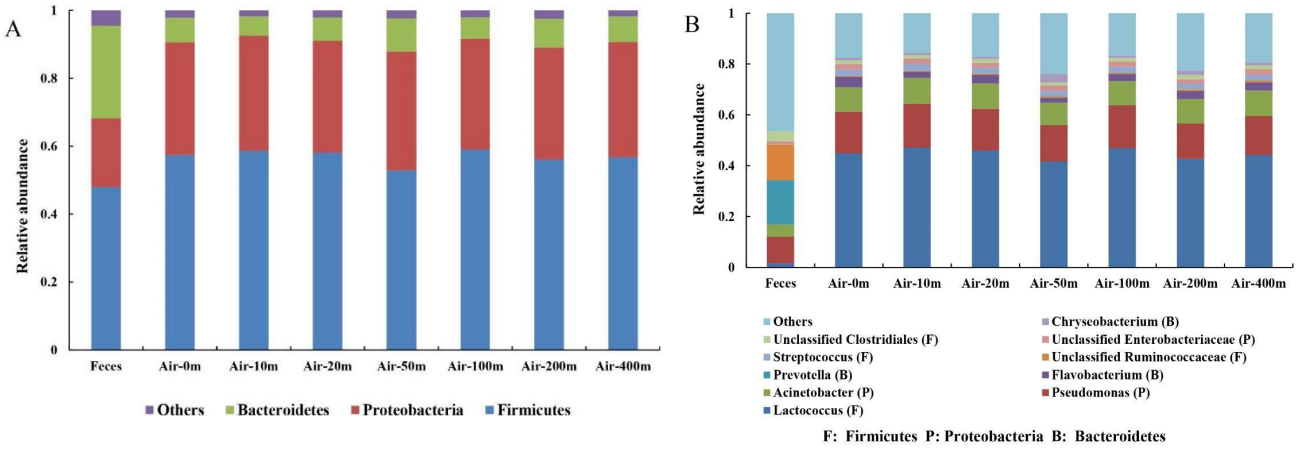

Fig. 1. Relative abundance of bacteria at the phylum (A) and Genus (B) levels in the faeces and air

3.2. Alpha and Beta diversity of bacteria

The richness and diversity of bacteria in faeces and air were analyzed by observed_species, Chao1, PD_whole_tree and Shannon statistical methods (Fig. 2). It was concluded that the bacterial diversity in faeces was apparently higher than in air ($P < 0.001$), while the similar diversity was found in the air at different distances. For instance, the Shannon index of faeces (8.05) was significantly ($P < 0.001$) higher than that for the air (average value 5.09), and there were no significant differences ($P > 0.05$) observed between Shannon indexes for air at each distance.

The beta diversity of bacteria in all samples was revealed by a weighted UniFrac distance matrix in the principal coordinates analysis (PCoA) diagram (Fig. 3). It could be concluded that there were apparent differences between the faeces and air samples (Fig. 3 A, C), based on the far distance in groups. However, no regularly located places were among the air samples collected at 0, 10, 20, 50, 100, 200 and 400 m from the swine barn (Fig. 3 B, D). Furthermore, the distance analyses indicated a great difference ($P < 0.001$) in the faeces-vs-air group, while all the air samples between distances didn't show significant ($P > 0.05$) change.

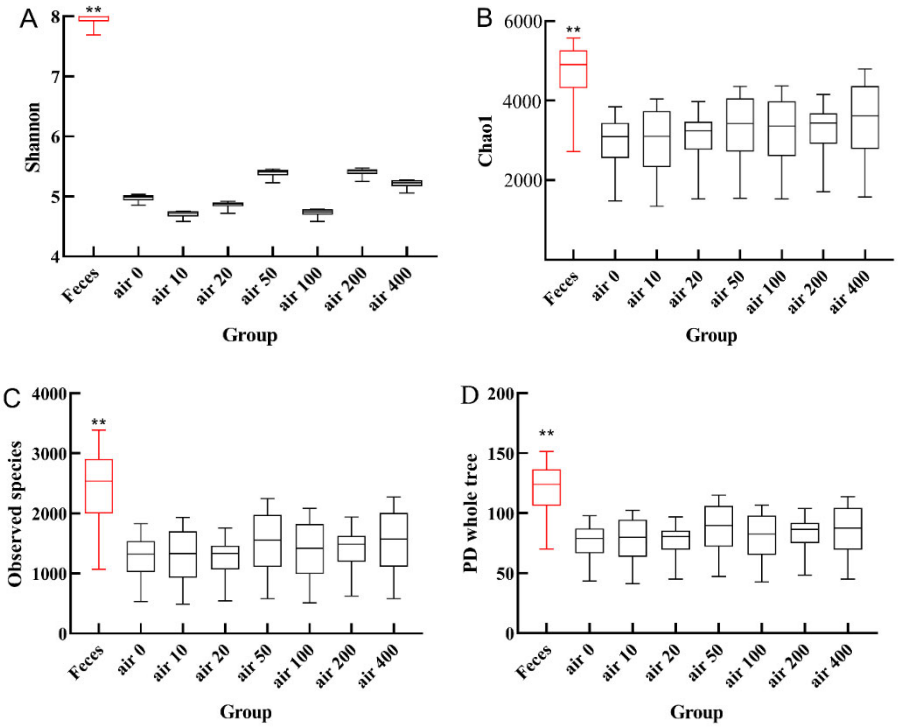

Fig. 2. The Shannon (A), observed_species (B), Chao1 (C) and PD_whole_tree (D) values in all samples

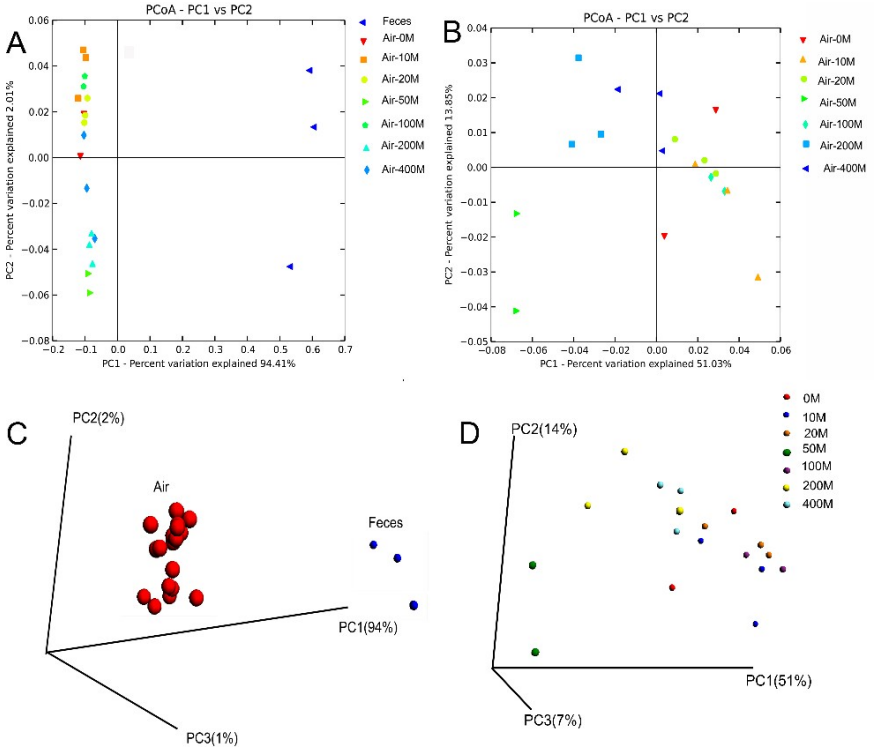

Fig. 3. The principal coordinates analysis (PCoA) diagram in all samples (A, C) and only air samples (B, D)

3.3. Sharing bacterial communities in air and faeces

The Venn diagrams were organized to measure all shared OTUs in faeces and air samples and are presented in Fig. 4. In total, 11214 and 6651 OTUs were detected in the air and faeces samples, respectively. The overlapped number was 4325 OTUs, which occupied 38% and 65% of the air and faeces samples, respectively. Moreover, 213 shared OTUs emerged after the OTUs clustering according to the Genus level. They were almost all of the faeces and 55% of the air. After clustering OTUs greater than 100 and 1000, the overlapped OTUs occupied more than 90% and 99% of the air, respectively. It means that the faeces and air share most of the bacteria, especially those in a dominant position.

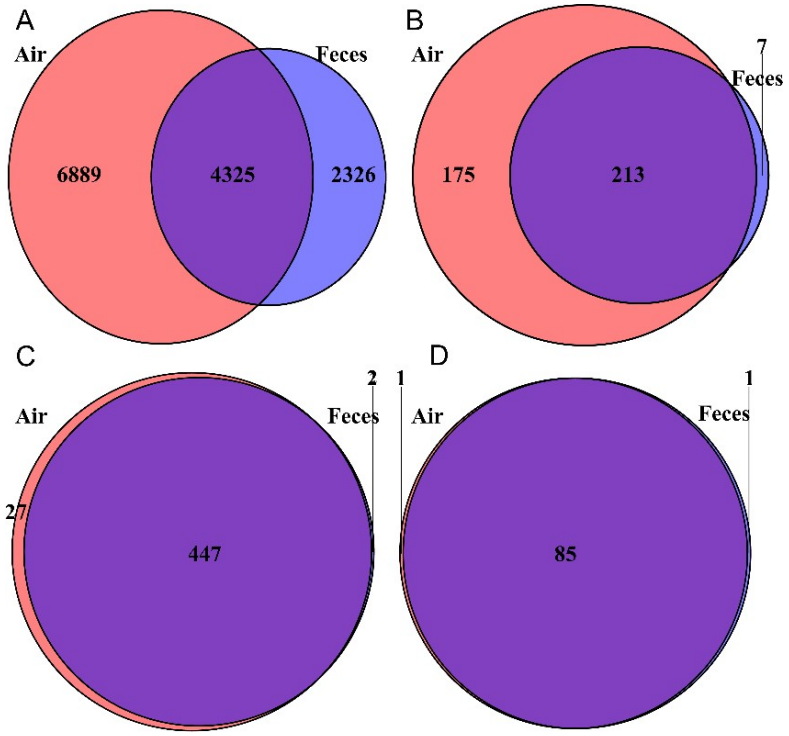

Fig. 4. The Venn diagrams of faeces and air samples by all OTUs (A); OTUs clustered according to the Genus level (B); OTUs clustered greater than 100 (C) and OTUs clustered greater than 1000 (D)

3.4. Pathogenic bacteria and their spreading

Six bacterial types (A, B, C1, C2, D and E) in all samples were classified by R software according to the abundance and existing site of OTUs (Table 2). A total of 132 OTUs were identified only in barn areas (C1), which means they do not cause pollution to the outside environment; 6689 OTUs inside the barn were less than outside (C2), which means they may come from the external environment. Especially the populations of 149 OTUs inside the barn were more changed than those outside, which were classified as type A. Therefore, it suggests that bacterial type A causes air pollution. In this way, the type B bacteria (1506 OTUs) have pollution potential as well cause their populations in the barn were higher than outside, even though they didn't change in a distance-dependent manner outside.

Table 2. The type and characteristics of bacteria at the Genus level

Bacterial Type	Characteristics	Total OTUs	Identified Genera
A	Bacteria in the barn were more than outside, and the populations changed in a distance-dependent manner	149	16
B	Bacteria in the barn were more than outside, and the populations did not change in a distance-dependent manner	1506	97
C1	Bacteria were detected only in the barn	132	17
C2	Bacteria outside the barn were more than inside	6689	261
D	Bacteria were detected in the faeces, specifically	969	35
E	Bacteria were detected in the air, specifically	436	67

It was shown that 149 OTUs (A) were detected at 10 m distance, while 15 and 1 OTUs were detected at 20 m and 50 m, respectively (Table S1). This result indicates that only 15 of them could be spread to 20 m away, and 1 of them can get 50 m distance. However, 16 identified genera can be dispersed to 10 m away at the genus level. Only *Prevotella* was detected at 20 m distance from the barn. The *Prevotella* was reported as a pathogen that may cause aspiration pneumonia and lung abscesses (Tanaka 2008). It indicated that hygienic interval in swine farm design is crucial, following Platz et al. (1995), who found that the airborne bacteria and dust decreased significantly with increasing distance in the range of 0-10 m. Moreover, Keessen et al. (2011) reported it is difficult to detect the bacteria Clostridium at up to 20 m distance. Still, this study detect-

ed the Clostridiales at the 50 m distance (Table S1). It was revealed that Clostridiales are predominant microbes that mediate psychiatric disorders (Li et al. 2020). The existence of opportunistic pathogens in airborne communities evidenced potential health risks to farmers and other residents from swine bioaerosol exposure. All the results above support the conclusion that the hygienic interval should be set more than 50 m from the barn to prevent spreading infectious diseases caused by airborne bacteria in swine facilities.

Furthermore, bacteria present explicitly in the faeces and the air were classified as type D and E, respectively (Table 2). A total of 969 OTUs were detected in type D, 35 genera were identified, and 20 were reported pathogens. In comparison, there were 436 OTUs detected in type E, 67 genera were identified, and 34 reported pathogens (Table S2). The pathogens with OTUs greater than 10 in type D were Prevotella, Parabacteroides, Treponema and Bacteroide. It was reported that the Prevotella is bad to lung, Parabacteroides is related to abdominal infection, Treponema can cause helicobacter bacteria diseases such as syphilis, nonvenereal syphilis, pinta and Yaws disease, and Bacteroides can cause abdominal infection (Tanaka 2008, Brook 2011, Knauf 2012, Cobo et al. 2021). The pathogens with OTUs greater than 10 in type E were Streptococcus which can cause septicemia, meningitis and pneumonia (Bonifait et al. 2014), Pseudomonas who generally affect lungs and urinary tract, or can cause burns and other blood infections (Todar 2006) and Chryseobacterium that can cause bacteremia (Dungan 2012). These results indicated that animal faeces are very important but not the only source of airborne pathogens. Therefore, it is necessary to take specific biological measures to control and prevent the spread of microorganisms.

4. Conclusions

It can be concluded from the results that the bacterial diversity in faeces was higher than in the air. Still, there were no significant differences among the air samples at different distances from the farm. The bacterial community compositions of faeces are differed from those in air, even though the faeces and air share most of the dominant bacteria. Moreover, pathogens present explicitly in the faeces and the air were Prevotella, Parabacteroides, Treponema, Bacteroide and Streptococcus, Pseudomonas, Chryseobacterium, respectively, animal faeces are only one of the source of airborne pathogens. Airborne pathogens Prevotella and Clostridiales could be spread 20 m and 50 m from the barn, respectively. So, the necessary hygienic distance of more than 50 m should be set to prevent spreading infectious diseases. Further research on the dispersion of prevalent pathogens in swine barns is needed.

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Supporting materials

Table S1. The spread of pathogens belonged to type A

Distance away from the farm									
	10 m			20 m			50 m		
Genus	OTUs	Pathogenic	Genus	OTUs	Pathogenic	Order	OTUs	Pathogenic
Prevotella	1	Yes (Tanaka et al.)	*Prevotella*	1	Yes (Tanaka et al.)	Clostridiales	1	Yes (Li et al.)
Bacteroides	3	Yes (Löfmark et al.)	/	/	/	/	/	/
Facklamia	1	Yes (Ananthakrishna et al.)	/	/	/	/	/	/
Carnobacterium	1	Yes (Leisner et al.)	/	/	/	/	/	/
Lactobacillus	1	Yes (Tanaka et al.)	/	/	/	/	/	/
Lactococcus	3	Yes (Zuily et al.)	/	/	/	/	/	/
Streptococcus	9	Yes (Hertzén et al.)	/	/	/	/	/	/
Ruminococcus	1	No	/	/	/	/	/	/
Blautia	2	No	/	/	/	/	/	/
Roseburia	1	No	/	/	/	/	/	/
Oscillospira	3	No	/	/	/	/	/	/
Veillonella	1	Yes (Huder et al.)	/	/	/	/	/	/
Bilophila	1	Yes (Baron et al.)	/	/	/	/	/	/
Acinetobacter	2	Yes (Hanski et al.)	/	/	/	/	/	/
Pseudomonas	1	Yes (Hardie et al.)	/	/	/	/	/	/
Akkermansia	1	Yes (Hardie et al.)	/	/	/	/	/	/
Others	117	/	/	/	/	/	/	/

Table S2. The bacteria and their pathogenic property of type D and E

D			E			E		
Genus	OTU	References	Genus	OTU	References	Genus	OTU	References
g_Fibrobacter	1	Madigan M.T. 2005	*g_Mycobacterium*	1	Hegedüs Z. 2009	*g_Kaistobacter*	1	
g_Staphylococcus	1	Hertzén E. 2012	*g_Friedmanniella*	1		*g_Paucibacter*	1	
g_Streptococcus	1	Hertzén E. 2012	*g_Butyricimonas*	1	Tyrrell K.L. 2011	*g_Janthinobacterium*	1	Lincoln S.P. 1999
g_02d06	1		*g_Prevotella*	1	Tanaka S. 2008	*g_Ralstonia*	1	
g_Roseburia	1		*g_Elizabethkingia*	1	Tuon F.F. 2007	*g_Methyloteneraa*	1	
g_Phascolarctobacterium	1		*g_Pedobacter*	1		*g_Bilophila*	1	Baron E.J. 1997
g_[Eubacterium]	1	Downes J. 2002	*g_Bacillus*	1	Madigan M.T. 2005	*g_Arcobacter*	1	Fera M.T. 2004
g_Bilophila	1	Baron E.J. 1997	*g_Peptoniphilus*	1	Citron D.M. 2012	*g_Psychromonas*	1	
g_Campylobacter	1	Gent R.N. 1999	*g_Clostridium*	1	Warny M. 2005	*g_Stenotrophomona*	1	Gilligan P.H. 2003
g_Succinivibrio	1	Somer H.J. 2002	*g_Anaerostipes*	1		*g_Synergistes*	1	
g_Lactobacillus	2	Tanaka S. 2008	*g_Propionibacteriu*	1		*g_Akkermansia*	1	Hardie K.R. 2009
g_Lactococcus	2		*g_Pseudonocardia*	1	Sen R. 2009	*g_Wautersiella*	2	Venkatachalam I. 2012
g_Blautia	2		*g_Porphyromonas*	1	Naito M. 2008	*g_Corynebacterium*	2	León C. 2004
g_Sutterella	2	Jyonouchi H. 2012	*g_Turicibacter*	1		*g_Methylobacterium*	2	
g_Acinetobacter	2	Hanski I. 2012	*g_Blautia*	1		*g_Achromobacter*	2	Duggan J.M. 1996
g_Pseudomonas	2	Hardie K R. 2009	*g_Coprococcus*	1		*g_Burkholderia*	2	Govan J.R. 1996
g_Sphaerochaeta	4	Ritalahti K.M. 2011	*g_Dorea*	1		*g_Psychrobacter*	2	Gini G.A. 1990
g_Coprococcus	6		*g_Dyadobacter*	1		*g_Bacteroides*	3	Brook I. 2011
g_Bulleidia	8	Kloesel B. 2013	*g_Brochothrix*	1		*g_Hymenobacter*	3	
g_Bacteroides	11	Brook I. 2011	*g_Cryocola*	1	Lacava P.T. 2007	*g_Lactobacillus*	4	Tanaka S. 2008
g_Treponema	13	Knauf S. 2012	*g_Roseburia*	1		*g_Oscillospira*	5	
g_Ruminococcus	14		*g_Parabacteroides*	1	Drew W.L. 2004	*g_Carnobacterium*	6	Leisner J.J. 2007
g_Parabacteroides	20	Drew W.L. 2004	*g_Microbacterium*	1		*g_Deinococcus*	6	
g_Prevotella	148	Tanaka S. 2008	*g_Arthrobacter*	1		*g_Sphingobium*	7	
g_rc4-4	2		*g_Megamonas*	1		*g_Acinetobacter*	7	Hanski I. 2012
g_YRC22	3		*g_Phascolarctobacte*	1		*g_Ruminococcus*	8	
g_Clostridium	1	Warny M. 2005	*g_Asticcacaulis*	1	Zheng D. 2007	*g_Flavobacterium*	9	Tuon F.F. 2007
g_Dorea	1	Mangin I. 2004	*g_Ochrobactrum*	1	Saeed Mahmood	*g_Chryseobacterium*	10	Kienzle N. 2001
g_L7A_E11	4		*g_Devosia*	1	M. 2000	*g_Pseudomonas*	10	Hardie K.R. 2009

Table S2. cont.

	D			E			E	
Genus	OTU	References	Genus	OTU	References	Genus	OTU	References
g_Coprobacillus	1		*g_Hyphomicrobium*	1	Rivas R. 2002	*g_Lactococcus*	11	
g_CF231	3		*g_Aminobacter*	1		*g_Streptococcus*	18	Hertzén E. 2012
g_Oscillospira	10		*g_Agrobacterium*	1		Others	270	
g_Lachnospira	2		*g_Paracoccus*	1	Hulse M. 1993			
g_p-75-a5	2	Hebb J.K. 2004	*g_Rhodobacter*	1	Dé I. 2004			
g_RFN20	3		*g_Roseomonas*	1				
Others	691		*g_Phaeospirillum*	1				

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