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Microbial ecology, bacterial pathogens, and antibiotic resistant genes in swine manure wastewater as influenced by three swine management systems☆

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ABSTRACT

The environmental influence of farm management in concentrated animal feeding operations (CAFO) can yield vast changes to the microbial biota and ecological structure of both the pig and waste manure lagoon wastewater. While some of these changes may not be negative, it is possible that CAFOs can enrich antibiotic resistant bacteria or pathogens based on farm type, thereby influencing the impact imparted by the land application of its respective wastewater. The purpose of this study was to measure the microbial constituents of swine-sow, -nursery, and -finisher farm manure lagoon wastewater and determine the changes induced by farm management. A total of 37 farms were visited in the Mid-South USA and analyzed for the genes 16S rRNA, *spaQ* (*Salmonella* spp.), Camp-16S (*Campylobacter* spp.), *tetA*, *tetB*, *ermF*, *ermA*, *mecA*, and *intI* using quantitative PCR. Additionally, 16S rRNA sequence libraries were created. Overall, it appeared that finisher farms were significantly different from nursery and sow farms in nearly all genes measured and in 16S rRNA clone libraries. Nearly all antibiotic resistance genes were detected in all farms. Interestingly, the *mecA* resistance gene (e.g. methicillin resistant *Staphylococcus aureus*) was below detection limits on most farms, and decreased as the pigs aged. Finisher farms generally had fewer antibiotic resistance genes, which corroborated previous phenotypic data; additionally, finisher farms produced a less diverse 16S rRNA sequence library. Comparisons of Camp-16S and *spaQ* GU (genomic unit) values to previous culture data demonstrated ratios from 10 to 10,000:1 depending on farm type, indicating viable but not cultivatable bacteria were dominant. The current study indicated that swine farm management schemes positively and negatively affect microbial and antibiotic resistant populations in CAFO wastewater which has future "downstream" implications from both an environmental and public health perspective.

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1. Introduction

Ecological adaptation, in any environment, is necessary for survival. Many factors influence bacterial adaptation within concentrated animal feeding operations (CAFOs); particularly, animal age and type, management (feeding and antibiotic use), and CAFO house design. Within a single industry, it is expected that animal age and animal management yield the most influence. Swine CAFOs can be separated into three stages or types based on age, each with their own animal and waste management: 1) sow (breeding, gestation, farrowing); 2) nursery (21 d–18 kg feeders); and 3) finisher farms (feeders to 113 kg) (McLaughlin et al., 2009). At each stage, animal management, including antibiotics, is carefully employed to sustain growth or treat infection (Sengelov et al., 2003; Rajic et al., 2006). Typically, swine liquid manure (e.g. wastewater) is land applied, which is essential to farm sustainability, regardless of farm stage (McLaughlin et al., 2009). Thus, each operation imposes its own selective pressures on gut microbiota and manure microbial populations; changes to antibiotic resistance, pathogens, and microbial ecology can serve as indicators, shedding light on agriculture's role in public and environmental health.

Some swine operators are shifting to focus on one developmental stage. This shift is largely dependent on market demands, centralized distribution, or environmental regulation. Nutrients, pathogens, and antibiotic resistance can be influenced by swine farm type (McLaughlin et al., 2009; Brooks and McLaughlin, 2009). Brooks and McLaughlin (2009) demonstrated a marked increase in antibiotic resistance from sow and nursery farms compared to finisher farms. A broad-range of antibiotics are administered on a large-scale basis, often in feed and water, throughout the pig rearing process, with a focus at early stages (Jindal et al., 2006; Rajic et al., 2006). Previous research only considered cultivated antibiotic resistant and pathogenic bacteria (Leung and Topp, 2001, Sengelov et al., 2003, Chinivasagam et al., 2004; Binh et al., 2008), though few studies measured genotypic resistance (Barkovskii et al., 2012; Chen et al., 2010). It is well known, that cultivation captures ~0.1% of bacteria; thus, potential antibiotic resistance, from both pathogenic and commensal bacteria, are uncounted. Quantitative polymerase chain reaction (qPCR), which is culture independent, overcomes this deficiency, yielding a more conservative quantification of microbial risks, which ultimately affects microbial risk assessment (Brooks et al., 2012).

Swine manure wastewater research recently has incorporated 16S rRNA fingerprinting, sequence libraries, and qPCR. Cotta et al. (2003), using a combination of culture and 16S rRNA gene sequencing, determined that lagoon wastewater was dominated by *Clostridium*, *Enterococcus*, and *Bacteroides*. Hog management and lagoon physicochemistry have led to temporal shifts in the microbial population (Cook et al., 2010; Lovanh et al., 2009). However, these studies focused on one swine farm type and offered no comparison based on farm type.

Therefore, the purpose of this study is to determine the effect of three different farm types: sow, nursery, and finisher farm management on select microbial populations of manure lagoon wastewater using culture-independent methodologies. The effect of farm management was quantified and qualified by targeting total eubacterial (i.e. 16S rRNA), antibiotic resistant, and bacterial pathogenic populations.

2. Materials and methods

2.1. Sample collection

Samples were collected in conjunction with previous studies (Brooks and McLaughlin, 2009; McLaughlin et al., 2009). Briefly, samples were collected from 37 anaerobic swine manure lagoons located in the Southeastern United States, from three different farm types, comprised of 17 sow, 10 nursery, and 10 finisher farms. Samples were collected at six locations per lagoon (three each on opposite lagoon sides) in sterile 250 ml polypropylene bottles using a modified PVC floatation float (McLaughlin et al., 2014). Sample aliquots were immediately frozen at −20 °C overnight and transferred to −65 °C for permanent storage.

2.2. DNA extraction

Frozen samples were thawed in a 25 °C waterbath prior to DNA extraction. Samples were processed by compositing 5 ml from each of 6 sub-samples, per lagoon, followed by microbial DNA extraction using a modified procedure employing the Mobio Power Soil DNA Extraction Kit (Mobio Laboratories, Inc.; Carlsbad, CA) and the Qiagen Qiamp DNA Stool kit (Qiagen; Valencia, CA). Briefly, the Power Soil kit was modified by removing lysis buffer from the bead beating tubes and replaced with 2 ml of the composited sample. The sample was then centrifuged at 20,000× *g* for 3 min and repeated three times, with interval ice steps. The supernatant was discarded at the final step, followed by addition of 450 μl of Qiagen ASL buffer and mixed by vortex. The entire volume was placed in a Fast Prep FP120 (Qbiogene, Inc.; Carlsbad, CA) at speed setting 5.0 for 20 s, and repeated three times with interval ice steps. Following shaking, a 20 μl lysozyme solution (65 mg ml^{-1}) was added to the mixture and incubated at 37 °C for 20 min, followed by 99 °C in a dry heat block for 10 min. The heated solution was vortexed for 15 s and centrifuged at 20,000× *g* for 1 min. The Qiagen Stool DNA extraction kit was then followed beginning with step 6 of the manufacturer's protocol.

2.3. PCR analysis

The resulting DNA was assayed for the presence of eubacterial, pathogenic, and antibacterial resistance genes: 16S rRNA (total eubacteria), *spaQ* (*Salmonella* spp.), Camp-16S (*Campylobacter*), *tetA*, *tetB*, *intI*, *ermA*, *ermF*, and *mecA* using qPCR. Primer pairs used in the study were as follows: 16S rRNA-16Sfor/rev (Nadkarni et al., 2002), *Salmonella* spp.-spaQF/R (Kurowski et al., 2002), *Campylobacter* spp.-campF2/R2 (Lund and Madsen, 2006), tetracycline resistance-tetAF/R and tetBF/R (Fan et al., 2007), class I integron-intIF/R (Hardwick et al., 2008), erythromycin resistance-ermAF/R and ermFF/R (Chen et al.,2010), and methicillin resistance-mecAF/R (Sabet et al.,

2007). The three-stage qPCR cycling conditions were as follows: one cycle at 95 °C for 10 min; 40 repetitions of two temperature cycles at 95 °C for 15 s, 60 °C for 1 min; followed by melt curve analysis at 95 °C for 15s, 60 °C for 30 s, and 95 °C for 15 s qPCR analyses were conducted in an ABI 7300 real-time system (Applied Biosystems, Foster City, CA) using ABI 96-well PCR plates, ABI qPCR-grade sealing film, and ABI syber green master mix. All samples were run in duplicate and amplification signals were quantified with a PCR-based standard curve and confirmed using melt-curve analysis. Standard control DNA was extracted from pure colony cultures using the Mobio Microbial DNA isolation kit. Standards were PCR-amplified, purified (Qiagen PCR Purification Kit), quantified, and serially diluted from 5×10^5 to 5 genomic unit per μl. Standard control isolates were as follows: 16S rRNA-*Escherichia coli* ATCC 25922 (American Type Culture Collection; Mansass, VA); *spaQ-Salmonella enterica* ATCC 14028; Camp-16S-*C.jejuni* ATCC 33560; *tetA*-tetracycline resistant environmental *E. coli* isolate; *tetB*-tetracycline resistant environmental *E. coli* isolate; *intI*-class I integron environmental *E. coli* isolate; *ermA*-erythromycin resistant environmental *Staphylococcus* spp. isolate; *ermF*-erythromycin resistant environmental *Staphylococcus* spp. isolate; and *mecA*-methicillin resistant *Staphylococcus aureus* ATCC BAA-1720. Sample results were reported as GU 100 ml^{-1} of swine effluent.

2.4. Clone library analysis

16S rRNA sequence clone libraries were prepared for sow, nursery, and finisher farm lagoons. Extracted DNA, from each lagoon, was first amplified with 16S rRNA primers, 8F and 1492R, using previously established conditions (Brooks et al., 2010). To avoid or at least limit amplification bias, three replicate sub-samples were separately amplified and combined using the Qiagen PCR purification kit prior to enzyme digestion. All reactions were carried out on a Thermo-Scientific Hybaid 0.2G thermocycler (Thermo-Scientific; Waltham, MA). To limit the number of libraries, lagoons were then selected based on amplified ribosomal DNA restriction analysis (ARDRA) (Massol-Deya et al., 1997). Briefly, each PCR product (500 ng) was subjected to enzyme restriction digestion using 3 units of RSA I (New England Biolabs; Ipswich, MA) and run on a Metaphor gel (3%) (Lonza Group Ltd; Switzerland) for 6 h at 100 V, using a modified ARDRA (Liu et al., 1997). Digest profiles were visually annotated and characterized using an AlphaImager with AlphaEase software V 4.0 (ProteinSimple; Santa Clara, CA). Lagoon profiles were manually compared to one another, and screened to remove duplicate or nearly identical bacterial community profiles within each farm type. A total of 9 clone libraries were then prepared. Purified 16S PCR products were then ligated into the Qiagen pDrive plasmid (Qiagen; Valencia, CA) and transformed into competent Qiagen *E. coli* cells using the Qiagen PCR cloning kit (Qiagen). Libraries comprised 5 sow, 2 nursery, and 2 finisher farms.

Individual clones were selected, amplified, and sequenced using both the 8F and 1492R primers. An ABI 3130xl Big Dye Terminator sequencing system (Applied Biosystems) was used for sequence reactions. Basecalls were viewed and edited using ABI Sequence Scanner (Applied Biosytems). Contiguous sequences (~1500 bp), prepared from forward and reverse reads, were generated via Sequencher v. 4.8 (Gene Codes Corporation; Ann Arbor, MI) using default settings.

Phylogenetic comparisons and sequence identifications were made using MOTHUR (Schloss et al., 2009) and the Ribosomal Database Project (Cole et al., 2003), respectively. Sequence libraries were composited into sow, nursery, and finisher lagoons and compared for differences in community richness, diversity, and structure. Approximately 900 clones were investigated, with approximately 530 used to construct libraries (sow = 292, nursery = 101, finisher = 128). Contiguous 16S rRNA sequences were compiled into individual libraries using Bioedit (Hall, 1999) and operational taxanomic units (OTUs) were assessed with MOTHUR at an OTU cutoff of 0.03. Sequence libraries were compared in pairwise fashion using libshuff and parsimony MOTHUR commands using a Tukey corrected *p* value of 0.0025. Individual libraries were subjected to MOTHUR rarefaction, chao-richness, and Shannon-diversity calculations to assess shared species-richness and diversity.

2.5. Statistics

Mean pathogen and antibiotic resistant qPCR levels were grouped by farm type and transformed to $\log_{10}$ values prior to statistical analyses. Geometric means were calculated for each farm type. A PROC MIXED model analysis was conducted using SAS Enterprise Guide 4.0 where qPCR levels (dependent variable) were compared based on swine farm type (class variable). Mean values were compared to ascertain differences between farm types. Residuals were normally distributed and the protected Fisher's least significant difference was used to determine significant pairwise differences.

3. Results and discussion

3.1. Pathogen genes

Analysis of 16S rRNA, pathogen, and antibacterial resistance genes revealed high values (>10^5 100 ml^{-1}) in at least one of every farm lagoon type (Fig. 1). The 16S rRNA gene levels in swine farm lagoon effluents was approximately 10^{11} GU 100 ml^{-1}, with statistically higher levels from finisher farms, followed by nursery and sows. These levels were similar to those obtained from farrowing facilities in other reported studies, approximately 10^{10}–10^{11} GU 100 ml^{-1} (Cook et al., 2010). To our knowledge, no study compares 16S rRNA levels throughout all three stages of swine production. Interestingly, finisher farms appeared to have the most stable and numerous bacterial populations, possibly as a result of fewer antibiotic inputs and an overall healthier/stabilized microbiota associated with older pigs. *Campylobacter* spp.16S rRNA (Camp-16S) levels were approximately 5–7 orders of magnitude less than the generic 16S rRNA measurements, described above. As with 16S rRNA, finisher farms were the most numerous, while sow farms had the least amount of *Campylobacter*-16S. Previous work conducted on these farms, demonstrated that *Campylobacter* (via culture most probable number assay [MPN]) were not statistically different when

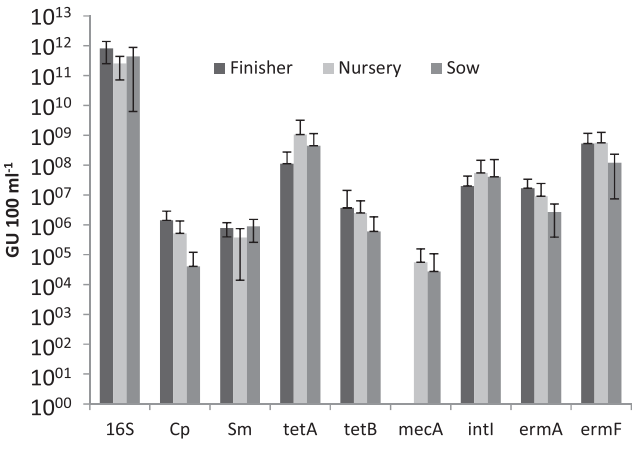

Fig. 1 – Influence of swine farm type on multiple bacterial genes. 16S – 16S rRNA; Cp – *Campylobacter* spp.; Sm – *Salmonella* spp.; tetA – tetracycline resistance A gene; tetB – tetracycline resistance B gene; mecA – methicillin resistance gene; intI – class 1 integron integrase gene; ermA – erythromycin resistance A gene; ermF – erythromycin resistance F gene.

means were compared across farm types (McLaughlin et al., 2009). However, *Campylobacter*-16S levels increased from 10^4 to 10^6 GU 100 ml^{-1}, sow to finisher farms, respectively; differences in levels may be due to selection imparted by swine management or by PCR inhibitors. Interestingly, these differences weren't noted in the previous study using culture analysis. The presence of *Salmonella* (*spaQ*) was not influenced by farm type, which corroborated previous culture analyses (McLaughlin et al., 2009).

Interestingly, Camp-16S and *spaQ* mean levels (for all three farms) were not statistically different (~10^6), while culture detection differed by nearly two orders magnitude (*Campylobacter* > *Salmonella*) (McLaughlin et al., 2009). This may indicate that *Campylobacter* is more suited for the pig or pig manure environment, with fewer propensities towards viable but not cultivatable (VBNC) cells. At the very least, this may indicate coextraction of DNA and/or PCR-inhibiting substances in sow and nursery farms which negatively influenced *Campylobacter* detection. Some *Campylobacter* assays were positive for culture but PCR-negative (data not shown), also indicating PCR inhibition or the result of increased sensitivity due to greater sample volume in the culture assay. In either instance, discrepancy in values can affect predictive risks (Brooks et al., 2012).

Table 1 – Molecular to culture ratios as influenced by swine farm type.

Farm type	Arithmetic mean GU:MPN ratio	
	Campylobacter	*Salmonella*
Sow	1.1×10^1:1	2.6×10^4:1
Nursery	9.1×10^1:1	1.2×10^4:1
Finisher	5.9×10^2:1	4.6×10^4:1
Mean	2.5×10^2:1	2.6×10^4:1

To investigate this, a GU to MPN ratio was developed using data from the current and previous studies, respectively (Table 1). The mean *Campylobacter* ratio was approximately 250:1, while *Salmonella* was approximately 26,000:1 GU:MPN, indicating a large discrepancy between qPCR and culture, particularly for *Salmonella*. Overall, the discrepancy between qPCR and culture techniques was more pronounced in finisher farms over nursery and swine farms, indicating a propensity towards viable but not cultivatable pathogens as the pig-rearing process continued. This may also suggest more inhibitory substances in sow and nursery farms. Ratios assumed that detection of GU was 1:1, meaning that one GU is equivalent to one cell, though it is well known that cell gene copy numbers vary, dependent on species or microbial metabolic state (Nadkarni et al., 2002). Therefore, the discrepancy in levels may be artificially high, though most likely not above a ratio of 10:1, indicating that VBNCs account for the remainder of the discrepancy and may become the physiological state of choice in some farm operations. This may have significant public health implications, regarding these operations and their land-applied fecal wastes, particularly if only cultivated values are reported or acted upon. For instance, it is well known that *Campylobacter* is prominent in poultry; however, *Campylobacter* is rarely detected in poultry litter using culture techniques (Brooks et al., 2010; Eberle et al., 2013; Roberts et al., 2014); however, using qPCR, Rothrock et al. (2008) demonstrated *Campylobacter* levels at ~10^3 g^{-1}. This phenomenon is expected with a cultivation-recalcitrant bacterium such as *Campylobacter*, given its growth requirements and fastidious nature. In the current study, *Salmonella* ratios were larger; therefore, it's possible that *Salmonella* readily enters a VBNC state under certain farm conditions, thus limiting accurate detection via culture. Additionally, it can't go without stating that continuous antibiotic use during any phase could select for VBNC or inactivate bacteria while DNA remains. Regardless of the reason, single reporting of either cultivated or molecular number may under- or over-estimate pathogen values, leading to misrepresentation of potential risk (Brooks et al., 2012).

3.2. *Antibiotic resistance genes*

All tested antibiotic resistance genes were found in at least one of each type of swine farm, except for finisher farms which did not have detectable levels of *mecA* (Table 2). mecA was the least prevalent antibiotic resistance gene with numbers ranging from below detection to 10^4 GU 100 ml^{-1}. Recently, reports have stated that pig products and farms contain methicillin resistant *S. aureus* (MRSA) (Casey et al., 2013; Smith et al., 2013); though to date, no study has demonstrated the presence of *mecA* genes or MRSA in swine manure lagoon effluent. In a previous study generic staphylococci levels (~10^6 CFU 100 ml^{-1}) in a commercial swine sow farm included in the current study remained stable throughout the year, regardless of season (McLaughlin et al., 2012). While no attempt was made to speciate the staphylococci, it is conceivable that a proportion could be *S. aureus*; further research is warranted to ascertain the level of *S. aureus*, and by extension MRSA, in swine manure lagoon effluent and its potential impact. The current study suggests

Table 2 – Frequency of detection for gene targets in lagoon effluent as determined by swine farm type.

FarmType	16S	Camp-16s.	*spaQ*	*tetA*	*tetB*	*mecA*	*intI*	*ermA*	*ermF*
Sow	17/17	9/17	16/17	17/17	9/17	2/17	17/17	16/17	17/17
	100%	53%	94%	100%	53%	12%	100%	94%	100%
Nursery	10/10	8/10	8/10	10/10	9/10	3/10	10/10	8/10	10/10
	100%	80%	80%	100%	90%	30%	100%	80%	100%
Finisher	10/10	10/10	10/10	9/10	7/10	0/10	9/10	9/10	9/10
	100%	100%	100%	90%	70%	0%	90%	90%	90%
Total	37/37	27/37	34/37	36/37	25/37	5/37	36/37	33/37	36/37
	100%	73%	92%	97%	68%	14%	97%	89%	97%

that mecA can number as high as 10^4 GU 100 ml^{-1} in nursery and sow manure lagoon effluent; however, the study also suggests that while *mecA* was detected in these farm types, finisher farms did not have detectable levels. This may indicate *mecA* resistance may be labile in both the pig and lagoon effluent once antibiotic pressure is removed. While pig farms do not use methicillin or closely related derivatives, it is conceivable that co-resistance could develop as a result of using other antibiotics (Monnet et al., 2004).

Overall, the highest level of antibiotic resistance GU was associated with *tetA* and *ermF* genes; *tetA* reached an average of 10^9 GU 100 ml^{-1} while *ermF* was approximately 6×10^8. Highest levels, for both genes, were reached in nursery farm lagoon effluent. While it wasn't possible to obtain antibiotic usage data from the currently evaluated farms, antibiotic use was consistent with other North American farms at the time. According to Dewey et al. (1997), USA farmers applied continuous antibiotics in ~75% of nursery feed which decreased to 55 and 44% for finishers and sows, respectively. Based on relatively recent reports, nursery, finishers, and sow farms employed various combinations of tetracycline, macrolide, penicillin, and aminoglycoside antibiotics depending on growth phase (Dewey et al., 1997; Apley et al., 2012). Dewey et al. (1997) reported that nursery and early stage growout farms were more likely to receive antimicrobials. Nurseries more often used apramycin and tetracycline/penicillin combinations; while, sows reported using tetracycline and neomycin combinations (Dewey et al., 1997).

tetA was the only gene found to be statistically influenced by farm type; however, when gene levels were normalized to 16S, the influence of swine farm type was noted for *tetA*, *ermF*, and *intI*, which may indicate an influence of cellular gene copy number. *tetB*, *intI*, and *ermA* ranged from 10^6 to 10^7 GU 100 ml^{-1}. It's not surprising to find tetracycline and macrolide resistance at such high levels in swine effluent as others have reported this trend (Barkovskii et al., 2012; Brooks and McLaughlin, 2009). Likewise, given the broad-based antibiotic usage in all three swine phases (Dewey et al., 1997), resistance levels would be expected to remain high. Barkovskii et al. (2012) reported various *tet* resistance alleles in three different stages of pig rearing with frequencies ranging from 1 *tet* GU per 10^3 to 10^7 16S rRNA GUs. These values are within the detectable range reported in the current study, indicating tetracycline resistance is common and widespread throughout pig rearing, regardless of process stage, geographic location, or antibiotic dose. Barkovskii et al. (2012) regarded tetracycline resistant genes as either transient or persistent based on below detection or persistence throughout all farm stages. Tetracycline resistance genes (*tetA* and *tetB*), in the current study, were readily detected throughout all farm types and individual farms. *tetB*, however, appeared to be the most "transient" as it was detected in 53, 90, and 70% of sow, nursery, and finisher farms, whereas *tetA* was detected in all lagoons (Table 2). Macrolide resistance genes (*ermA* and *ermF*) were detected in nearly all lagoons with 89 and 97% of positive detections in lagoons, respectively. Similar findings have been reported for other swine farms (Chen et al., 2010). Rajic et al. (2006) reported antibiotic use in Canadian swine operations stating chlorotetracycline (tetracycline class) and tylosin (macrolide class) were the most used antibiotics in weaner and finisher operations, respectively, which would corroborate the current results. The same study demonstrated that, overall, fewer farms used antibiotics as pig age increased (nursery > finisher > sow). Though, an American study estimated that gross usage of antibiotics increased with age (Apley et al., 2012), partially due to the higher pig weights. The discrepancies in these two studies demonstrates the difficulties in assessing antibiotic use on farm. In the current study, it would appear that antibiotic resistance genes were found in nearly all swine lagoons, a propensity towards younger pigs was apparent.

The integrase gene (*intI*), from class I integrons, was also widely distributed throughout all swine farm types and lagoons. Integrase was present and found in the same proportions as *tetA* and *ermF*, possibly indicating co-selection; integrons are commonly associated with multi-antibiotic resistance (MAR) in a variety of environments (Leverstein-van Hall et al., 2002). Previous investigation from these farms indicated numerous instances of MAR among isolates, particularly from nursery and sow farms (Brooks and McLaughlin, 2009). It is important to note, that only a small fraction of potential resistance genes were investigated and thus, interpretations could change with a more exhaustive analysis.

3.3. 16S rRNA sequence libraries

The effect of farm type on the microbial members of each swine manure lagoon was investigated. Specific clone libraries from lagoons were selected, to avoid replication, by comparing 16S ARDRA restriction profiles. Based on ARDRA discrimination, it appeared that sow farms (5 unique profiles) were more diverse than nursery or finisher farms. Nursery and finisher farms only presented two unique profiles each (data not shown), which may indicate that continuous antibiotic use limited variability between farms. The libraries

were compared with Mothur and an overall trend emerged from libshuff and parsimony analyses suggesting that 16S rRNA OTU libraries from finisher farms were significantly different from nursery and sow farms (data not shown), though parsimony analysis suggested all three swine manure lagoons were significantly different from one another. Single library chao estimations (alpha diversity) indicated sow farms were the most enriched, followed by finisher and nursery farms. Once again, this may indicate decreased antibiotic use. Shared chao and ace estimations demonstrated that shared richness was highest between nursery and sow farms, while finisher farms shared less (Fig. 2). Estimates of dissimilarity (sorest and thetayc) demonstrated finisher farms were more dissimilar to nursery and sow farms, than comparisons between nursery and sow farms (Fig. 2). As can be seen from Fig. 3, the distribution of identified sequences at the phylum level showed that Bacteroidetes, Proteobacteria, and Firmicutes were the most dominant phyla in all three swine farm lagoons (Fig. 3a). Sow farms demonstrated the most diverse distribution of phyla with 8/9 phyla represented; closer inspection of represented classes reveals 16/18 classes were represented in sow farms, followed by nursery farms (Fig. 3b). Despite more sequences represented from sow farms, their distributions were weighted in classes already heavily represented in nursery and finisher farms (Clostridia and Gammaproteobacteria), indicating Proteobacteria and Firmicutes were dominant phyla. Though dominant taxa remained the same throughout all three farm type stages, there was a subtle loss in diversity as pigs aged, indicating decreasing diversity among microbial inhabitants of the swine manure lagoons as pigs age and the fecal, hence intestinal, microbial biota stabilizes. It also may suggest a decrease in potential disease, which may lead to fewer disturbances to gut microbiota. Cook et al. (2009) reported on the distribution of 16S rRNA genes in a swine farrowing operation manure lagoon. Their findings indicated that dominant sequences were distributed among two phyla, Bacteroidetes and Firmicutes, a finding also reflected in the current study. Cook et al. (2009) also indicated that taxa were seasonally dependent, and that the genus *Bacteroides* was particularly interesting as a correlation between malodorous compounds and the taxa was found, particularly in the winter. Though the current study did not investigate seasonal variation, the Bacteroidetes class, of which Bacteroides belongs to, was more dominant in nursery farms, indicating potential for increasing malodorous releases from these swine farm types. Goh et al. (2009) reported similar findings to those presented here. It is important to note that though 500 sequences were evaluated, rarefaction analysis of each library (data not shown) demonstrated that a more exhaustive sequencing endeavor is warranted.

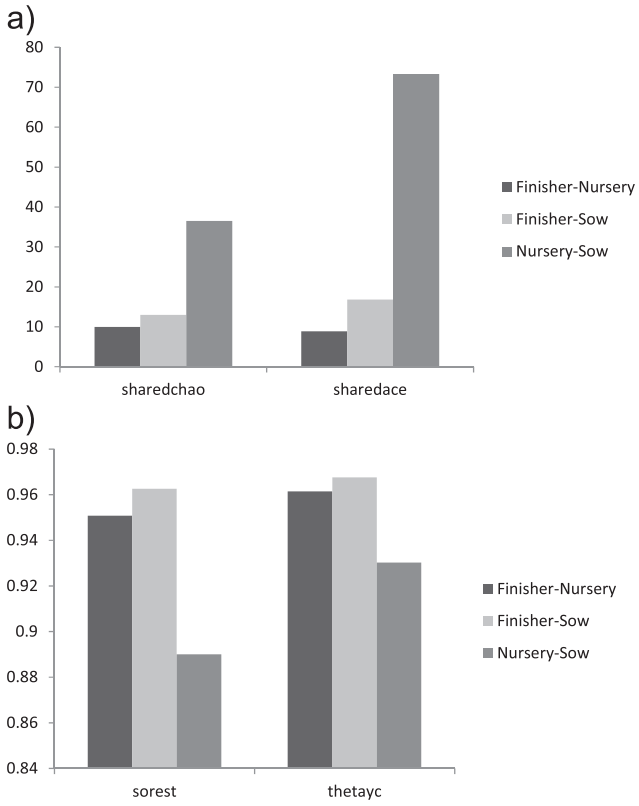

Fig. 2 – a) Shared bacterial community richness (chao & ace estimation) and b) dissimilarity in bacterial community membership (Sorensen estimation [sorest]) and structure diversity (Yue & Clayton theta [thetayc]) estimates between each farm type combination.

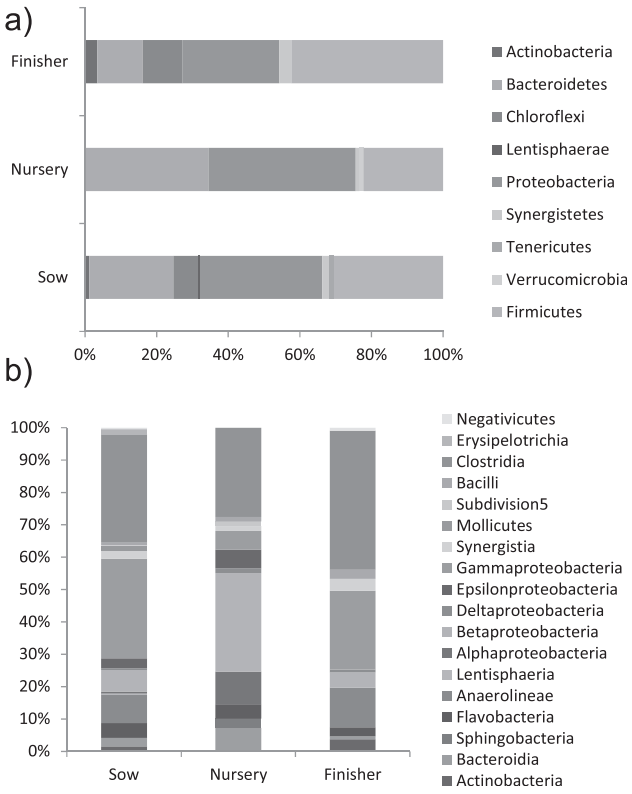

Fig. 3 – Distribution of 16S rRNA sequences based on swine farm type classified by a) phylum, and b) class.

4. Conclusions

This study represents a unique genomic microbial comparison of swine farm type, including: sow, nursery, and finisher

operations. While studies of this kind have been conducted in the past, most have focused on one type of pig-rearing operation or focused on culture methods to facilitate comparisons. Economic burdens and demands of environmental protection have put pressures on the swine industry, in certain parts of the country, which may dictate the regional dominance of specific swine farm management types.

- In the current study, finisher farms were found to be the most unique operation, in nearly all measured aspects.
- Tetracycline and erythromycin resistance were abundantly found in all three types of wastewater, while the current findings suggest swine wastewater is not a major source of the methicillin resistance gene, *mecA*.
- Finisher farms harbored the fewest antibiotic resistance genes, and represented the least diverse microbial ecology. Finisher farms are known to continuously use antibiotics in feed and thus may lead to decreased diversity; however, fewer antibiotic resistance genes may indicate less therapeutic treatment, a shift to other resistance genes, or a stabilizing gut microbiota.
- A shift to finisher farms appears to also select for *Campylobacter* and *Salmonella*, while also selecting for VBNC *Campylobacter*, as the discrepancy between GU and culture MPN increased in finisher farms.
- Sow and nursery farms, statistically, had the most antibiotic resistance, while their microbial ecologies were the most similar.
- Reliance on cultivated or genomic values, solely, under- or over-estimates reported pathogen values; therefore, it's advised that both values be evaluated from a risk perspective.

The implications associated with shifting industry priorities are unknown, though it is certain that swine CAFOs will continue to land apply their manure lagoon effluent, as it is currently the most economical and environmental viable approach to treat this wastewater. However, the presence of antibiotic resistance genes and pathogens still require environmental stewardship on the part of farm operations, particularly when protecting application site borders (e.g. limited public exposure and rain runoff).

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