Metagenomics of Antimicrobial and Heavy Metal Resistance in the Cecal Microbiome of Fattening Pigs Raised without Antibiotics

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ABSTRACT This study aimed to assess the cecal microbiome, antimicrobial resistance (AMR), and heavy-metal resistance genes (MRGs) in fattening pigs raised under antibiotic-free (ABF) conditions compared with ordinary industrial pigs (control, C) using whole-genome shotgun sequencing. ABF pigs showed enrichment of *Prevotella* (33%) and *Lactobacillus* (13%), whereas *Escherichia coli* (40%), *Fusobacterium*, and *Bacteroides* (each at 4%) were notably observed in the C group. Distinct clusters of cecal microbiota of ABF and C pigs were revealed; however, microbiota of some C pigs (C1) appeared in the same cluster as ABF and were totally separated from the remaining C pigs (C2). For AMR genes, the most abundant genes, *tet(Q)* (35.7%) and *mef(A)* (12.7%), were markedly observed in the ABF group, whereas *tet(Q)* (26.2%) and *tet(W)* (10.4%) were prominently shown in the C group. *tet(Q)* was positively correlated with *Prevotella* in ABF and C1 samples. In the C2 group, the prominent *tet(W)* was positively correlated with *Fusobacterium* and *Bacteroides*. The pigs studied here have never received tetracycline, but pregnant sows received chlortetracycline once every 7 days before parturition. Chromosomal Cu and Zn resistance genes were also shown in both groups regardless of the received Cu and Zn feed additives. A higher abundance of multimetal resistance genes was observed in the C group (44%) than with the ABF group (41%). In conclusion, the microbiome clusters in some C pigs were similar to that in ABF pigs. High-abundance tetracycline resistance genes interrelated to major bacteria were observed in both ABF and C pigs. MRGs were also observed.

IMPORTANCE AMR is an increasing problem in farm animals, and raising farm animals without antibiotics is one method that could solve this problem. Our study showed that only some tetracycline and macrolide resistance genes, *tet(Q)*, *tet(W)*, and *mef(A)*, were markedly abundant in ABF and C groups. The *tet(Q)* and *tet(W)* genes interrelated to different predominant bacteria in each group, showing the potential role of major bacteria as reservoirs of AMR genes. In addition, chromosomal Cu and Zn resistance genes were also observed in both pig groups, independent of the use of Cu and Zn additives on both farms. The association of MRGs and AMR genotypes and phenotypes, together with the method to resensitize bacteria to antibiotics, should be studied further to unveil the cause of genes conferring high-level resistance and solve these problems.

KEYWORDS antimicrobial resistance genes, microbiome, pig, raised without antibiotics, whole-genome shotgun sequencing, pigs

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The pig industry and its marketing have grown rapidly in response to the growing demand for high-quality, low-cost animal protein. In 2030, the world pig population is expected to be 1,062 million, having risen from 873 million in 1997/1999 (1). In pig production, a number of factors (e.g., pathogens, stress, and inappropriate nutrition and environment) can cause diseases leading to economic loss (2, 3). In pigs, the study of the microbiome, particularly in the gastrointestinal (GI) tract, using metagenomic approaches can be related to systemic infections, growth development, and disease resistance (4, 5). Metagenomic analysis of the V3-V4 region of the 16S rRNA gene in the GI tract of pigs was conducted previously and reported that *Lactobacillus* and *Clostridium* were the major genera in the small intestine, whereas *Prevotella* was the most abundant in the large intestine (6). A comparative study of the microbial community in different portions of pig intestinal tract by 16S rRNA gene sequencing revealed that microbial composition in cecum and colon were significantly different from that in ileum. The microbiome in cecum and colon of pigs with high feed conversion ratios played an important role in producing short-chain fatty acids and indolic compounds required for the system of feed conversion ratios (7).

The use of antibiotics might alter gut microbiota, which could lead to an increased reservoir of antimicrobial resistance (AMR). Subtherapeutic doses of antibiotics were previously applied on porcine farms as a growth promoter, a practice that was subsequently prohibited in Europe, the United States, Canada, and Thailand. Presently, antibiotics are used for a short term at therapeutic doses to prevent, control, and treat diseases (8). However, antimicrobial consumption in food animals could generate a pervasion of AMR in the supply chain, affecting public health (9, 10). Extended-spectrum β -lactamase (ESBL)-harboring *Escherichia coli* was found in ceca from pigs at a slaughterhouse in the United Kingdom (11). In addition, ESBL/AmpC/carbapenemase-positive *E. coli* was reported in fattening pigs in Germany, and carbapenemase-positive *E. coli* was detected in pig meat in Belgium (12). Tetracycline-resistant coliforms were detected in fecal samples from nursery pigs treated with oxytetracycline for *Lawsonia intracellularis*-induced diarrhea in Denmark (13). High levels of AMR genes have been reported due to the use of high levels of tetracycline and macrolide in pig farms (14).

Following the increased concerns by consumers of AMR and drug residue contamination in pork products, an approach of rearing animals without antibiotics was introduced (15). Ampicillin- and tetracycline-resistant *E. coli* was isolated before the pre-weaning period in antibiotic-treated pigs at higher rates than in the pigs raised under antibiotic-free (ABF) conditions (16). In Thailand, the Department of Livestock Development has launched a campaign for antibiotic-free livestock production, which corresponds to the national food safety strategy to reduce antibiotics usage in livestock to 30% within 5 years (2017 to 2021) according to the Thailand National Strategic Plan on Antimicrobial Resistance 2017 to 2021 (17). In commercial pig production, Cu and Zn supplements are usually formulated in feed additives to promote growth (18, 19). In addition, Zn has been used to prevent diarrhea, improve antioxidants, and reduce intestinal inflammation in weaning piglets (20–23). An understanding of microorganism diversity relating to AMR and heavy-metal resistance genes (MRGs) is needed for the development of safe and sustainable pig production and pork safety.

The objective of this study was to compare microbiomes and the changes in the abundances of AMR genes and MRGs from cecal contents of fattening pigs raised with and without antibiotic treatment in their life cycles using whole-genome metagenomics.

RESULTS

Microbial diversity and relative abundance analysis. The total number of microbial species in the ABF group was shown to be lower than that in the C group; however, from the Shannon diversity index, the increased species in the C group were not evenly distributed (Fig. 1). Taxonomic classification at the phylum level of cecal microbes with $\geq 0.01\%$ abundance and with different distributions of microbial

FIG 1 Box-and-whisker plot with individual data points for species richness (A) and Shannon diversity index values (B) of fattening pigs raised under antibiotic-free conditions (ABF) and the ordinary industrial system (C). *, $P < 0.001$.

communities between ABF and C groups is shown in Fig. 2A. Higher abundance of *Bacteroidetes* and *Firmicutes* was observed in the ABF group, whereas the abundance of *Proteobacteria*, *Fusobacteria*, and *Actinobacteria* was significantly increased in the C group (Fig. 2B). A principal coordinate analysis (PCoA) plot showed obviously divergent clusters of the cecal microbiota between ABF and C groups; however, microbiota of some C pigs appeared in the same cluster as ABF pigs (C1 group) and totally separated from the remaining C pigs (C2 group) (Fig. 3). In addition, hierarchical clustering of the cecal microbiome at the genus level showed similar patterns of ABF and C1 groups, which were different from that of the C2 group (Fig. 4). Differently abundant taxa were then identified using linear discriminant analysis (LDA) combined with the LDA effect size (LEfSe) algorithm. Microbes with LDA scores of ≥ 3 in the ABF group included *Prevotella*, *Megasphaera*, *Mitsuokella*, *Faecalibacterium*, *Parabacteroides*, *Afipia*, *Sutterellaceae_unclassified*, *Coprococcus*, *Butyrivibrio*, *Roseburia*, *Erysipelotrichaceae_unclassified*, and *Dorea*, whereas *E. coli*, *Bacteroides*, *Fusobacterium*, *Desulfobacterium*, *Sutterella*, and *Methanobrevibacter* were enriched in the C group (Fig. 5A). The LEfSe cladogram revealed differentially abundant genera of the cecal microbiome between the ABF and C groups (Fig. 5B). The enriched genera with $\geq 1\%$ abundance in the ABF group included *Prevotella* (33.76%), *Lactobacillus* (13.11%), *Megasphaera* (2.97%), *Parabacteroides* (2.79%), *Faecalibacterium* (2.25%), *Mitsuokella* (2.12%), and *Afipia* (1.89%), whereas *E. coli* (40.85%), *Fusobacterium* (4.56%), *Plesiomonas* (4.56%), *Bacteroides* (4.07%), *Desulfovibrio* (1.71%), and *Parabacteroides* (1.53%) were more concentrated in the C group (Fig. 5B; also see Table S1 in the supplemental material).

Global AMR gene diversity and abundance analysis. Antimicrobial drug resistance patterns were plotted. The tetracycline resistance pattern was the most abundant in both ABF and C groups. ABF pigs had a higher proportion of AMR genes in the macrolide class; in contrast, AMR genes in aminoglycosides, a group of phenicols, lincosamides, oxazolidinones, pleuromutilin, and streptogramin A, and the other 14 antimicrobial drug resistance patterns ([i] colistin, [ii] fluoroquinolone, [iii] fosfomycin, [iv] macrolide, aminoglycoside, tetracycline, fluoroquinolone, phenicol, and rifampin, [v] macrolide, lincosamide, and streptogramin B, [vi] metronidazole [5-nitroimidazole], [vii] oxazolidinone and phenicol, [viii] phenicol, [ix] quinolone, [x] rifampin, [xi] streptogramin B, [xii] sulfonamide, [xiii] trimethoprim, and [xiv] vancomycin), appeared to be enriched in the C group (Fig. 6). Differential relative abundance of AMR genes was

FIG 2 Relative abundance of cecal microbial communities at the phylum level of fattening pigs raised under antibiotic-free conditions (ABF) and the ordinary industrial system (C). (A and B) Stacked bar plot showing taxonomic relative abundance distribution (A) and box-and-whisker plot showing relative abundance of phyla, with statistically significant differences indicated (B). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

then displayed using LDA coupled with the LEfSe algorithm (24). Genes with LDA scores of ≥ 3 in the ABF group included *tet(Q)*, *mefA*, *erm(F)*, *tet(O/32/O)*, *cfxA*, and *erm(G)*, whereas *tet(W)*, *cfr(C)*, *ant(6)-Ia*, *aph(3'')-III*, *tet(W/32/O)*, *lnu(C)*, *tet(A)*, *mdf(A)*, *bla_{TEM-1B}*, *tet(O/W)-1*, *sul2*, *aph(6)-Id*, *aph(3'')-Ib*, *floR*, *tet(M)*, *bla_{OXA-85}*, and *ant(3'')-Ia* were enriched in the C group (Fig. 7A). The LEfSe cladogram revealed differentially abundant AMR genes between ABF and C groups (Fig. 7B). The enriched genes with $\geq 1\%$ abundance in the ABF group included *tet(Q)* (35.69%), *mefA* (12.67%), *erm(F)* (5.44%), *tet(O/32/O)* (2.92%), *cfxA* (2.88%), and *tet(44)* (2.20%), whereas *tet(W)* (10.43%), *cfr(C)* (4.82%), *aph(3'')-III* (4.47%), *tet(O)* (3.56%), and *tet(W/32/O)* (2.21%) were more concentrated in the C group (Fig. 7B, Table S2).

FIG 3 Principal coordinate analysis (PCoA) plot of cecal microbiome taxonomy at the genus level obtained from fattening pigs raised under antibiotic-free conditions (ABF) and the ordinary industrial system (C). The oval shows a C sample group that is separated from the ABF group.

Correlation of bacterial community structure, samples, and AMR gene abundance. Permutation tests showed three canonical correlation analysis (CCA) plots with statistical significance, including tetracycline, lincosamide, and phenicol and lincosamide, oxazolidinone, pleuromutilin, and streptogramin A resistance patterns (Fig. 8). According to the CCA plot of a tetracycline resistance pattern, highly abundant (>1%) *tet(Q)* and *tet(O/32/O)* in the ABF and C1 groups were interrelated to the predominant bacteria, *Prevotella*, *Lactobacillus*, *Faecalibacterium*, *Megasphaera*, and *Mitsuokella*. In contrast, the C2 group was cognate to highly abundant *tet(W/32/O)*, *tet(W)*, and *tet(O)*, which were interrelated to *Fusobacterium* and *Bacteroides* (Fig. 8A, Table S3). For the CCA plot of a lincosamide resistance pattern, *InuC*, found in both ABF and C groups, was interrelated to *Plesiomonas*, *Fusobacterium*, *Bacteroides*, and *Desulfovibrio* (Fig. 8B, Table S3). In addition, highly abundant *cfr(C)*, of the phenicol, lincosamide, oxazolidinone, pleuromutilin, and streptogramin A resistance patterns, was relevant to *Plesiomonas* (Fig. 8C, Table S3).

Global MRG diversity and abundance analysis. In our study, a statistically significant higher abundance of the multimetal resistance gene group, the most abundant group, was observed in the C group (approximately 44%). Genes that were enriched in the C group included the cation efflux system protein CusA (*cusA*), zinc/cadmium/lead-transporting P-type ATPase (*zntA*), silver exporting P-type ATPase (*silP*), silver efflux pump SilA (*silA*), etc. Most of them encoded efflux pump proteins. The second-ranked groups were a Cu resistance gene group (approximately 13%) and a drug, biocide, and metal resistance group (approximately 14%). Genes with high abundance (>1%) included coatomer subunit alpha (*copA*), blue copper oxidase CueO (*cueO*), apolipoprotein N-acyltransferase (*cutE*), copper resistance protein A (*pcoA*), multidrug resistance protein MdtC (*mdtC*), multidrug resistance protein MdtB (*mdtB*), aminoglycoside efflux pump (*acrD*), multidrug resistance protein MdtA (*mdtA*), and signal transduction histidine-protein kinase BaeS (*baeS*). Other heavy metals presenting resistance at 5 to 6% abundance included Zn, Ni, and As. The level of the sodium resistance gene group was shown to be higher in the ABF group, with the abundance in both ABF and C groups being approximately 3% (Tables 1 and 2).

DISCUSSION

The present study showed different abundances and the correlation of the microbiome and AMR genes in fattening pigs raised under antibiotic-free conditions compared with

FIG 4 Hierarchical clustering of cecal microbiome at the genus level of fattening pigs raised without antibiotics (A), raised under ordinary industrial system group 1 or C1 (B), and raised under ordinary industrial system group 2 or C2 (C).

ordinary industrial pigs. Although farms for ABF and C pigs were separated and far apart, their pregnant sows received similar treatments, and both groups were the same breeds and had the same rearing system (except for the drugs indicated in Table 3); hence, this might contribute to only moderate differences of microbiome and AMR in the ABF and C groups. However, according to the percent relative abundance with or without LDA scores in our study, the ABF and C groups showed different bacterial communities. *Prevotella* (33%) and *Lactobacillus* (13%) were the major genera in the ABF group, whereas *E. coli* (40%) *Fusobacterium* (4%), *Plesiomonas* (4%), and *Bacteroides* (4%) were major genera in the C group. In a previous study, the enriched *Prevotella* was shown to be associated with plant-based carbohydrate degradation (25). ABF pigs received probiotics containing

FIG 4 (Continued)

Lactobacillus spp. at an age of 1 to 3 days to improve growth performance, intestinal morphology, antioxidant status, immune system, and gut health. For the intestinal microbiome, probiotics were fed to enhance *Lactobacillus* and *Bifidobacterium* and reduce *E. coli* and *Enterococcus* (26). In our study, ABF pigs contained more populations of *Lactobacillus* and fewer of *E. coli* than the C group. A previous study in weaned pigs that received in-feed chlortetracycline, sulfamethazine, and penicillin at subtherapeutic doses for 14 days showed a markedly increased *E. coli* population in feces (27). A massive population of commensal *E. coli* was not preferable for the farm, because it can acquire mobile genetic elements by horizontal gene transfer and, hence, become a major reservoir for antimicrobial resistance- and virulence-associated genes, such as *dfrA*-, *aadA*-, *cmlA*-, and *sul*-containing

FIG 4 (Continued)

integrans (28, 29). This implies that commensal *E. coli* can be used as an indicator species in AMR surveillance, because its AMR phenotype corresponded to the drug used. Increased AMR genes, either in abundance or diversity, have been shown in treated pigs, although some enriched genes were not relevant to the administered drugs (27).

In our study, we also found high abundance of tetracycline resistance genes, although chlortetracycline was used only once for pregnant sows. In fact, chlortetracycline and oxytetracycline have been minimally used in pig farms in Thailand compared with enrofloxacin and penicillin-streptomycin on both small (<50 sows)- and

FIG 5 (Continued)

FIG 5 LEfSe analysis of the cecal microbiome of fattening pigs raised under antibiotic-free conditions (ABF) and the ordinary industrial system (C). (A) Histogram of the LDA scores indicating differential abundances of microbial taxa between the ABF and C groups with an LDA of ≥ 2 . (B) Cladogram showing the phylogenetic distribution and differentially abundant genera of the cecal microbiome by phyla of ABF and C groups. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

medium (100 to 500 sows)-scale farms (30, 31). Nonetheless, resistance to tetracycline was highly observed in *E. coli* isolates from pigs and contact and noncontact humans in Northeastern Thailand (31, 32). At an AMR gene level, we found the predominant *tet(Q)* gene more frequently in the ABF group than the C group. According to the CCA plot, *tet(Q)* interrelated with *Prevotella*, the major bacterium in the ABF group, and this might be a reason why *tet(Q)* was shown more often in the ABF group. Pregnant sows receiving tetracycline during the pregnancy period might be another reason why tetracycline resistance genes were maintained in the environment. We also observed *erm(F)* and *cfxA* in ABF pigs. The *tet(Q)* and *erm(F)* genes are commonly located on the same conjugative transposons and could confer resistance to tetracycline, macrolides, lincosamides, and streptogramin B (MLS_B) (33). The *tet(Q)*, *erm(F)*, and *cfxA* genes have been reported in *Prevotella* strains isolated from humans with periodontitis, cystic fibrosis, and other diseases and in healthy humans (34–37).

The microbiome in the C group could be divided into C1 and C2 clusters. The C1 cluster was similar to the ABF group (Fig. 3). The reason for this remains unclear, as the ABF and C farms are far apart and pigs were sent to the slaughterhouse at different times (approximately 4 h apart). High-abundance *tet(W)* and its mosaic, *tet(W/32/O)*, were interrelated to the enriched *Fusobacterium* and *Bacteroides*. The *tet(W)* gene has been reported in feces of weaned pigs receiving oxytetracycline in-feed at 10 mg/lb of body weight daily for 7 days (38). The C pigs received tulathromycin (macrolide class), amoxicillin (β -lactam class), tylvalosin (macrolide class), tiamulin (pleuromutilin class), halquinol, organic Cu, and nano Zn but not tetracycline or its derivatives. However, pregnant sows in the C group received the same treatments as those in the ABF group, which might lead to the maintenance of tetracycline resistance genes. In addition, we found higher *mef(A)* gene levels in

FIG 6 Relative abundance of AMR genes by antimicrobial drug resistance patterns of fattening pigs raised under antibiotic-free conditions (ABF) and the ordinary industrial system (C). (A) Stacked bar plot showing relative abundance distribution of AMR genes by antimicrobial drug resistance patterns. (B) Box-and-whisker plot showing relative abundance of AMR genes by antimicrobial drug resistance patterns and statistical differences. $n=20$. *, $P < 0.001$.

the ABF group than the C group. ABF pigs received neither tulathromycin nor tylvalosin. However, pregnant sows were treated with tylvalosin for 14 days during the parturition period (Table 3). Enterococci carrying the *mef(A)* gene were previously reported in feces from healthy pigs (39). The dissemination of macrolide-resistant genes to the environment via pig manure is concerning (40). We found *aph(3')-III* (aminoglycoside class), which was also relevant to *E. coli*, *Fusobacterium*, and *Bacteroides*. Its closely related gene, *aph(3')-IIIa*, has been reported to be harbored by *E. coli* reservoirs (36). In addition, *cfr(C)* was found to be interrelated to *Plesiomonas*. The use of florfenicol led to the enrichment of *Plesiomonas* and increased *cfr(C)* (41, 42). However, there is no obvious

FIG 7 (Continued)

FIG 7 LEfSe analysis of AMR genes of fattening pigs raised under antibiotic-free conditions (ABF) and the ordinary industrial system (C). (A) Histogram of the LDA scores indicating differential abundances of AMR genes between ABF and C groups with an LDA of ≥ 2 . (B) Cladogram showing the distribution of AMR genes and differential abundance by antimicrobial drug resistance patterns of ABF and C groups. *, adjusted P value (P_{adj}) of < 0.05 ; **, $P_{adj} < 0.01$; ***, $P_{adj} < 0.001$.

relationship between *Plesiomonas* and *cfr*(C). We also observed higher levels of *bla*_{CTX-M-55}, encoding CTX-M-55 extended-spectrum beta-lactamases (ESBLs), in the C group than the ABF group, possibly associated with the use of amoxicillin in the C group. Bacteria harboring plasmid-mediated *bla*_{CTX-M-55} have been shown to be multiple drug resistant (MDR) and rapidly disseminated (43). Higher MICs of tetracycline, clindamycin, and amoxicillin have been reported in *tet*(Q)-, *erm*(F)-, and *cfxA*-positive *Prevotella* isolates, respectively; however, the presence of AMR genes might not reflect a high susceptibility to antimicrobial resistance. The *erm*(F) gene in *Prevotella* isolates from human clinical specimens did not lead to clindamycin or erythromycin resistance phenotypes (35–37). Furthermore, *cfxA* detected in *Prevotella* species isolates did not contribute to cephamycin resistance (44). This was possibly owing to the presence of nonfunctional AMR genes or the gene products not providing resistance at a functional level (45). Hence, it is possible that the AMR genotypes in our study do not reflect their phenotypic resistance. The limitation of the present study is the lack of phenotypic confirmation of resistance, which needs to be performed with the fresh cecal samples but not the frozen ones.

Heavy metals such as Cu and Zn, used in feed formulations, and other heavy metal contaminants (Zn, Cd, and Hg), found in fecal waste or in food production, have been reported to be associated with copper and zinc resistance genes and β -lactam resistance genes (46). In our study, we found *pcoA*, *silA*, *silS*, and *silP* were carried by plasmids and possibly affected by Cu and Zn formulated in feed additives. Cu and Zn were used as feed additives at higher levels (10 to 250 mg/kg of body weight and 125 to 3,000 mg/kg, respectively) than the dietary requirement on pig farms to promote growth and prevent diarrhea from colibacillosis (18, 47). *silA*, *silS*, and *silP* are composed of the *sil* operon, whereas *pcoA* is a part of the *pco* copper resistance operon (48, 49). *sil* and *pco* gene clusters have been found to be located on a Tn7-like transposon in *E. coli* (50, 51), and *E. coli* acts as a reservoir of

FIG 8 Canonical correspondence analysis (CCA) plot showing the distribution of fattening pigs raised under antibiotic-free conditions (ABF), ordinary industrial system groups 1 and 2 (C group 1 and C group 2, respectively), microbiome genera, and AMR genes according to corresponding antimicrobial drug resistance patterns. (A) CCA plot of AMR genes corresponding to tetracycline drug resistance pattern. (B) CCA plot of AMR genes corresponding to lincosamide drug resistance pattern. (C) CCA plot of AMR genes corresponding to phenicols, lincosamides, oxazolidinones, pleuromutilins, and streptogramin A drug resistance patterns. Abbreviations: *Acidaminococcus* (G1), *Aeromonas* (G2), *Afipia* (G3), *Alistipes* (G4), *Bacteroides* (G5), *Brachybacterium* (G6), *Brevibacterium* (G7), *Butyrivibrio* (G8), *Catenibacterium* (G9), *Cetobacterium* (G10), *Clostridium* (G11), *Collinsella* (G12), *Coprococcus* (G13), *Corynebacterium* (G14), *Dermabacteraceae* unclassified (G15), *Dermatophilaceae* unclassified (G16), *Desulfovibrio* (G17), *Dietzia* (G18), *Dorea* (G19), *Enterococcus* (G20), *Erysipelotrichaceae* unclassified (G21), *Escherichia* (G22), *Eubacterium* (G23), *Faecalibacterium* (G24), *Fusobacterium* (G25), *Lactobacillus* (G26), *Megasphaera* (G27), *Mitsuokella* (G28), *Oxalobacter* (G29), *Parabacteroides* (G30), *Phascolarctobacterium* (G31), *Plesiomonas* (G32), *Prevotella* (G33), *Roseburia* (G34), *Streptococcus* (G35), *Sutterella* (G36), *Sutterellaceae* unclassified (G37), *tet(Q)* (T1), *tet(W)* (T2), *tet(O)* (T3), *tet(O/32/O)* (T4), *tet(W/32/O)* (T5), *tet(X)* (T6), *tet(O/W)* (T7), *tet(O/W)-2* (T8), *tet(A)* (T9), *tet(32)* (T10), *tet(M)* (T11), *tetA(P)* (T12), *tet(B)* (T13), *tet(X4)* (T14), *tetB(P)* (T15), *tet(X3)* (T16), *tet(Z)* (T17), *tet(G)* (T18), *tet(O/W/O)-3* (T19), *tet(O/W)-1* (T20), *tet(33)* (T21), *tet(C)* (T22), *tet(S/M)* (T23), *Inu(A)* (L1), *Inu(B)* (L2), *Inu(C)* (L3), *Inu(D)* (L4), *Inu(F)* (L5), *Inu(G)* (L6), *Inu(P)* (L7), *cfr(C1)* (C2), *cfr(C)* (C3). An asterisk indicates a statistically significant difference between ABF and C groups.

pco-sil genes and could transfer them to *Salmonella enterica*, leading to higher copper resistance in *S. enterica* (51). In addition, the association between *pco* and *tet(B)* has been observed in *E. coli* isolated from weaned piglets (52). However, most of the heavy-metal resistance genes we observed were integrated into the bacterial chromosome (<https://www.uniprot.org/>). The most abundant genes were in a multimetal resistance group, which showed a high abundance of genes encoding proteins associated with transporters or efflux pumps of several heavy metals, such as Mg, Ag, Fe,

FIG 8 (Continued)

and Zn. In a drug, biocide, and metal resistance group, ranking second in MRG abundance were the *mdtA*, *mdtB*, and *mdtC* components of the *mdtABC* operon, encoding MdtABC multidrug efflux pumps, while *baeS* and *baeR* are in the *baeSR* operon, the regulator of MdtABC drug efflux systems, and are transcribed together with *mdtABC* as a monocistronic RNA in *E. coli* and *Salmonella* (53, 54). We also found components of the *nik* operon, *nikA*, *nikB*, *nikC*, *nikD*, and *nikE*, in addition to *nikR*, encoding NikR, a regulatory protein of the *nik* operon in *E. coli*. The *nik* operon is required for Ni uptake into bacterial cells (55, 56). For the As resistance, *pstA*, *pstB*, *pstC*, and *pstS* were observed. These genes are related to a phosphate transporter, as the phosphate analogue arsenate has been shown to enter microbial cells via a phosphate transporting system (57). Since these AMR genes are located on chromosomes, they appeared regardless of the antimicrobial or metal used. In addition, the coselection of heavy-metal resistance genes and AMR genes has been reported (58). This might be why we observed some high-abundance AMR genes in ABF pigs that have never been given antimicrobial drugs.

However, in the present study, we did not see a relationship between MRGs and antimicrobial drug resistance patterns (data not shown). The relationships between heavy metals, MRGs, and AMR at the genotype and phenotype levels should be investigated further.

FIG 8 (Continued)

Conclusions. This study revealed that the microbiome clusters in some C pigs were similar to those in ABF pigs. Only some of the highly abundant tetracycline resistance genes were observed and interrelated to major bacteria in ABF and C groups, although chlortetracycline was used once in pregnant sows in the last trimester but not in the fattening pigs in either group. Higher abundances of multimetal resistance genes, the most plentiful MRGs, were observed in the C group compared with the ABF group. Chromosomal Cu and Zn resistance genes were also shown in both groups at high abundance, independent of the use of excess Cu and Zn feed additives. Hence, longer withdrawal periods may be required, and blocking the specific gene expression might be an alternative strategy to solve the AMR problem.

MATERIALS AND METHODS

Sample collection. Forty samples of cecal contents were obtained from a private slaughterhouse located in Chonburi, Thailand. Twenty samples each were from crossbred Landrace × Large White × Duroc Jersey fattening gilts and barrows at 23 weeks, either raised under antibiotic-free conditions (ABF group) or with antibiotics by following the farm's program per veterinary advice (C group). Fattening pigs weighed 90 to 120 kg. Both ABF and C groups were reared under the same farming contract. Farms for ABF and C pigs were separated and far apart. The distance between ABF and C farms is approximately 245 km. The ABF farm has raised only ABF pigs for 2 years (4 generations). Their pregnant sows received similar treatments. Pigs were reared in farrowing pens until weaning and transferred to wean-to-finish pens in the other farms. The feeding program for pregnant sows and ABF and C pigs is shown in Table 3. In the case of illness in the ABF group, pigs received Nutriphenol (tannin, ≥73.5%) (Nutri-Concept, Fougères, France) to treat diarrhea, AgroVit MBL (an

TABLE 1 Relative abundance ($\geq 1\%$ in at least one group) of MRGs in pigs raised under ABF or an ordinary industrial system

Metal resistance pattern	Gene	Relative frequency (%) $\pm$ SD for group:		Adjusted <i>P</i> value
		ABF	C	
Drug and biocide and metal resistance	<i>mdtC</i>	3.34 $\pm$ 0.81	3.40 $\pm$ 0.43	0.75
Drug and biocide and metal resistance	<i>mdtB</i>	3.34 $\pm$ 0.94	3.44 $\pm$ 0.40	0.26
Multimetal resistance	<i>cusA</i>	3.18 $\pm$ 0.86	3.70 $\pm$ 0.55	0.17
Copper resistance	<i>copA</i>	3.17 $\pm$ 0.89	2.77 $\pm$ 0.39	0.30
Multimetal resistance	<i>zntA</i>	2.99 $\pm$ 0.74	3.22 $\pm$ 0.53	0.40
Multimetal resistance	<i>mgtA</i>	2.95 $\pm$ 1.12	2.85 $\pm$ 0.80	0.89
Drug and biocide and metal resistance	<i>acrD</i>	2.79 $\pm$ 1.57	2.97 $\pm$ 0.61	0.94
Copper resistance	<i>cueO</i>	2.37 $\pm$ 0.74	2.24 $\pm$ 0.71	0.79
Multimetal resistance	<i>pitA</i>	2.28 $\pm$ 0.80	1.87 $\pm$ 0.33	0.22
Multimetal resistance	<i>silP</i>	2.26 $\pm$ 0.97	3.20 $\pm$ 1.30	0.25
Nickel resistance	<i>nikA</i>	1.94 $\pm$ 0.64	1.61 $\pm$ 0.49	0.30
Zinc resistance	<i>zraR</i>	1.91 $\pm$ 0.36	1.90 $\pm$ 0.30	0.68
Multimetal resistance	<i>silA</i>	1.83 $\pm$ 1.21	2.45 $\pm$ 0.98	0.49
Copper resistance	<i>cutE</i>	1.48 $\pm$ 0.55	1.71 $\pm$ 0.30	0.62
Multimetal resistance	<i>silS</i>	1.40 $\pm$ 0.87	1.84 $\pm$ 0.82	0.43
Arsenic resistance	<i>pstS</i>	1.39 $\pm$ 0.39	1.42 $\pm$ 0.24	0.43
Multimetal resistance	<i>cusB</i>	1.37 $\pm$ 0.35	1.33 $\pm$ 0.21	0.99
Multimetal resistance	<i>mntH</i>	1.35 $\pm$ 0.43	1.25 $\pm$ 0.20	0.30
Nickel resistance	<i>nikB</i>	1.35 $\pm$ 0.43	1.09 $\pm$ 0.39	0.39
Sodium resistance	<i>nhaA</i>	1.35 $\pm$ 0.45	1.11 $\pm$ 0.35	0.30
Arsenic resistance	<i>pstC</i>	1.31 $\pm$ 0.43	1.39 $\pm$ 0.22	0.36
Multimetal resistance	<i>cusS</i>	1.26 $\pm$ 0.52	1.48 $\pm$ 0.25	0.65
Drug and biocide and metal resistance	<i>mdtA</i>	1.25 $\pm$ 0.39	1.32 $\pm$ 0.19	0.62
Multimetal resistance	<i>fiE</i>	1.24 $\pm$ 0.42	1.07 $\pm$ 0.18	0.36
Arsenic resistance	<i>pstA</i>	1.22 $\pm$ 0.56	1.37 $\pm$ 0.26	0.87
Drug and biocide and metal resistance	<i>baeS</i>	1.18 $\pm$ 0.67	1.44 $\pm$ 0.14	0.68
Arsenic resistance	<i>pstB</i>	1.17 $\pm$ 0.68	1.10 $\pm$ 0.22	0.82
Sodium resistance	<i>nhaB</i>	1.11 $\pm$ 0.66	1.06 $\pm$ 0.59	0.92
Multimetal resistance	<i>zraS</i>	1.11 $\pm$ 0.39	1.12 $\pm$ 0.14	0.65
Copper resistance	<i>pcoA</i>	1.07 $\pm$ 0.97	1.20 $\pm$ 0.73	0.77
Multimetal resistance	<i>corA</i>	1.04 $\pm$ 0.55	1.31 $\pm$ 0.21	0.40
Nickel resistance	<i>nikC</i>	1.02 $\pm$ 0.42	0.88 $\pm$ 0.26	0.56

acidifier) (Agromed, Cairo, Egypt) to treat *Streptococcus* infection at week 8, Aromax (essential oils) (Afrimash, Oyo, Nigeria) for coughing and respiratory problems, and Bio-Complex (vitamins) (TT & D Products, Pathum Thani, Thailand) for fever instead of antibiotics. Samples were kept at -20°C until further analysis.

DNA preparation and metagenomic sequencing. DNA from 200 mg of frozen cecal contents was extracted and purified using a QIAamp Fast DNA stool minikit (Qiagen, Hilden, Germany) and a DNA

TABLE 2 Relative abundance of metal resistance genes by metal resistance groups of fattening pigs raised under ABF or the ordinary industrial system

Metal resistance group	Relative frequency (%) $\pm$ SD for group:		<i>P</i> value ^a
	ABF (<i>n</i> = 20)	C (<i>n</i> = 20)	
Arsenic	5.55 $\pm$ 1.16	5.69 $\pm$ 0.82	0.44
Biocide and metal	6.27 $\pm$ 1.47	5.73 $\pm$ 1.08	0.16
Chromium	0.63 $\pm$ 0.28	0.61 $\pm$ 0.19	0.51
Copper	13.47 $\pm$ 2.99	12.99 $\pm$ 1.96	0.97
Drug, biocide and metal	14.84 $\pm$ 2.18	14.69 $\pm$ 0.98	0.66
Iron	0.91 $\pm$ 0.86	0.95 $\pm$ 0.89	0.59
Mercury	0.64 $\pm$ 0.73	0.37 $\pm$ 0.53	0.14
Multimetal	41.12 $\pm$ 3.06	44.40 $\pm$ 3.36	0.00**
Nickel	6.70 $\pm$ 1.26	5.61 $\pm$ 1.61	0.09
Sodium	3.71 $\pm$ 0.66	3.09 $\pm$ 0.99	0.03*
Tellurium	0.00 $\pm$ 0.01	0.01 $\pm$ 0.02	0.71
Zinc	6.16 $\pm$ 0.90	5.87 $\pm$ 0.49	0.38

^a*, $P < 0.05$; **, $P < 0.01$.

TABLE 3 Feeding program for pregnant sows and fattening pigs raised under ABF or the ordinary industrial system

Feed, probiotics, and drug(s)	Route	Age	ABF	C
Sows				
400 ppm chlortetracycline at day −7 to −6 (day 0 = parturition day)	Oral (feed)		✓	✓
100 ppm tylosin at day −7 to 7	Oral (feed)		✓	✓
10 mg/kg kanamycin at day 1–3	Intramuscular		✓	✓
Piglets				
Probiotics (Bactosac, K.M.P. Biotech, Chon Buri, Thailand) containing 10 ⁹ CFU each of <i>Bacillus subtilis</i> , <i>Bacillus licheniformis</i> , <i>Lactobacillus acidophilus</i> , <i>Lactobacillus plantarum</i> , <i>Pediococcus pentosaceus</i> , and <i>Saccharomyces cerevisiae</i>	Oral	Day 1–3	✓	
15 mg/kg amoxicillin and 2.5 mg/kg tulathromycin	Intramuscular	Week 1 (castration)		✓
15 mg/kg amoxicillin and 2.5 mg/kg tulathromycin	Intramuscular	Week 3 (3 days before weaning)		✓
150 ppm tylosin, 400 ppm amoxicillin, Zn (nano) and Cu (organic)	Oral (feed)	Week 6–8 for 7–10 days		✓
200 ppm tiamulin, 240 ppm halquinol, Zn (nano) and Cu (organic)	Oral (feed)	Week 13–15 for 7–10 days		✓
Zn (organic) and Cu (inorganic)	Oral (feed)	Week 13–15 for 7–10 days	✓	

Clean & Concentrator-10 kit (Zymo Research, Irvine, CA, USA) by following the manufacturers' instructions. Genomic DNA concentration and quality were ascertained using a NanoDrop ND 1000 spectrophotometer V3.7 (Thermo Fisher Scientific, Waltham, MA, USA) and 1% agarose gel electrophoresis staining with RedSafe nucleic acid staining solution (iNtRON Biotechnology, Gyeonggi, South Korea). Forty genomic DNA samples at 20 ng/μl with A_{260}/A_{280} absorbance ratios of 1.8 to 2.0 were submitted to Novogene (Beijing, China) for shotgun metagenomic DNA sequencing. Before shotgun metagenomic sequencing, DNA purity, DNA degradation, potential contamination, and quantification were determined by Novogene. Metagenomic DNA libraries were constructed and sequenced using 150-bp paired-end sequencing on the Illumina NovaSeq 6000 platform (Illumina, San Diego, CA, USA) at 10-GB whole-metagenome shotgun (WMS) data output.

Data analysis. Raw sequences were quality controlled and preprocessed using FASTP version 0.20.0, a FASTQ data preprocessing tool (<https://github.com/OpenGene/fastp/>; accessed 3 March 2020) (59). Mismatched base pairs in overlapped regions of paired-end reads were corrected. Low-quality bases at the 5' and 3' ends, poly(G) at 3' ends, and adaptors were trimmed. Pruning reads containing >10% undetermined nucleotides (N), bad reads, and low-quality bases (average Phred score of <20) were filtered out (59).

The marker-based taxonomic profiling was constructed at different levels using MetaPhlAn2 version 2.69.1 (<https://anaconda.org/bioconda/metaphlan2/>; accessed 3 March 2020). The cleaned metagenomic reads were aligned against marker genes presented in microbial reference genomes via the nucleotide aligner Bowtie2 version 2.3.4 in the MetaPhlAn2 package (60). The alpha diversity of samples was evaluated using species richness and the Shannon diversity index, which were calculated from the genus proportion using the vegan package version 2.5–6 (<https://CRAN.R-project.org/package=vegan/>; accessed 10 March 2020) in the R program (version 4.0.2) (<https://cran.r-project.org/>; accessed 10 March 2020). For beta diversity, relative abundance of microbial community across samples, defined as a percentage of the total read counts in a sample, was generated from MetaPhlAn2 data using Microsoft Excel 2019 (61). A principal coordinate analysis (PCoA) plot was constructed from the microbial community composition in each sample based on Bray-Curtis dissimilarities via the pcoa plot function in RAM package version 1.2.1.7 (<https://CRAN.R-project.org/package=RAM/>; accessed 10 March 2020) of the R program. LDA coupled with the LEfSe algorithm version 1.0 (<https://huttenhower.sph.harvard.edu/galaxy/>; accessed 6 April 2020) was used to reveal the bacterial distribution associated with ABF and C groups based on the relative abundance values with an LDA score of >2.0 (60). A cladogram, which reveals differentially abundant taxa of a microbial community, was constructed and annotated by Interactive Tree of Life (iTOL), version 5.6.3 (<https://itol.embl.de/>; accessed 6 April 2020) (62). The phylogenetic tree in the cladogram was produced from the representative 16S rRNA sequences of each genus using Molecular Evolutionary Genetics Analysis version 7.0 (MEGA7) software (<https://www.megasoftware.net/>; accessed 6 April 2020) (63). A heat map of bacterial abundance in each group at the genus level was constructed with the pheatmap (version 1.0.12) (<https://CRAN.R-project.org/package=pheatmap>; accessed 10 May 2020) in R package.

To acquire information for AMR genes and antimicrobial drug resistance patterns, the cleaned metagenomic data were aligned with available AMR reference genes in the ResFinder version 3.2 database (<https://cge.cbs.dtu.dk/services/ResFinder/>; accessed 3 March 2020) using robust insert size estimates (64). Metagenome annotation, such as AMR gene families, drug classes, resistance mechanisms, antibiotics, etc., was performed using the Comprehensive Antibiotic Resistance Database (CARD) 2020 databases (<https://card.mcmaster.ca/>; accessed 17 August 2020) (41). The stacked bar chart of relative frequency of AMR accumulation in antimicrobial drug resistance patterns of an individual sample was initiated by the proportion of read counts in each sample from the ResFinder annotation (downloaded 3 March 2020).

and presented using Microsoft Excel. Differential abundance of AMR genes corresponding to antimicrobial drug resistance patterns between ABF and C groups was analyzed using the DESeq2 version 1.30.0 package (<https://bioconductor.org/packages/release/bioc/html/DESeq2.html>; accessed 17 August 2020) in R software, and data were visualized using the ggplot2 version 3.2.1 in tidyverse package (<https://CRAN.R-project.org/package=tidyverse/>; accessed 17 August 2020) of the R program. A PCoA plot was constructed from the AMR genes in each pig based on Bray-Curtis dissimilarities via the pcoa function in R. LDA combined with the LEfSe revealed the AMR genes associated with ABF and C groups based on the relative abundance values with an LDA score of >2.0 . A cladogram of differential AMR gene abundance analysis was created and annotated from the read counts of each AMR gene using iTOL (62). The tree in the cladogram was initiated from antimicrobial drug resistance patterns of each gene by the ResFinder annotation. CCA depicted the association of bacterial community structure at the genus level, pig samples, and antimicrobial drug resistance patterns using the vegan R package. Its significance was assessed via a permutation test under a reduced model.

For MRG analysis, a stacked bar chart of relative frequency of MRG accumulation in metal class level of individual samples was constructed by the proportion of read counts in each sample from MEGARes v. 2.0.0 (<https://megares.meglab.org>; accessed 10 September 2020) (65) and presented using Microsoft Excel. Differential abundance of MRGs between ABF and C groups was analyzed using DESeq2 in the R package. CCA was conducted to describe the association of bacterial community structure at the genus level, pig samples, and metal classes in addition to the association of antimicrobial drug resistance patterns, pig samples, and metal classes using the vegan R package.

Statistical analysis. Before data analysis, the Shapiro–Wilk test was used to verify the homogeneity of variance. Statistical analyses of species richness, Shannon diversity index, relative abundance of microbial phyla and microbial genera, and MRG relative abundance of metal class were performed by Mann–Whitney U test. Pearson's chi-squared test was applied to show any significant difference in phylum distributions between two groups. Adonis in R was used to test the null hypothesis of no difference of microbiome diversities between ABF and C groups. *P* values of <0.05 indicated statistical significance.

Data availability. Metagenomic sequences were deposited in the Sequence Read Archive (SRA) at NCBI under the accession number [PRJNA694380](https://www.ncbi.nlm.nih.gov/sra/PRJNA694380).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

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G.S., N.A., R.C., and T.L. designed the study. P.T. and G.S. performed the experiments. P.T., W.M., and S.T. analyzed the data. R.C., T.L., P.T., and G.S. wrote the final manuscript.

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