Shifts in microbial community, pathogenicity-related genes and antibiotic resistance genes during dairy manure piled up

Xu Zhang,1,2† Chenjie Ma,1,2‡ Wen Zhang,1,2 Wu Li,1,2 Jialin Yu,1,2 Di Xue,1,2 Xiaolin Wu1,2,*‡ and Guangcun Deng1,2,†
1Key Lab of Ministry of Education for Protection and Utilization of Special Biological Resources in Western China, Ningxia University, Ningxia, China.
2School of Life Science, Ningxia University, Ningxia, China.

Summary
The uncomposted faeces of dairy cow are usually stacked on cow breeding farms, dried under natural conditions and then used as cow bedding material or they may be continuously piled up. However, no information is available to evaluate variations in the human and animal pathogen genes and antibiotic resistance during the accumulation of fresh faeces of dairy cow to manure. Here, we present the metagenomic analysis of fresh faeces and manure from a dairy farm in Ning Xia, showing a unique enrichment of human and animal pathogen genes and antibiotic resistance genes (ARGs) in manure. We found that manure accumulation could significantly increase the diversity and abundance of the pathogenic constituents. Furthermore, pathogens from manure could spread to the plant environment and enphytonic pathogens could affect the yield and quality of crops during the use of manure as a fertilizer. Levels of virulence genes and ARGs increased with the enrichment of microbes and pathogens when faeces accumulated to manure. Accumulated manure was also the transfer station of ARGs to enrich the ARGs in the environment, indicating the ubiquitous presence of environmental antibiotic resistance genes. Our results demonstrate that manure accumulation and usage without effective manure management is an unreasonable approach that could enrich pathogenic microorganisms and ARGs in the environment. The manure metagenome structure allows us to appreciate the overall influence and interaction of animal waste on water, soil and other areas impacted by faecal accumulation and the factors that influence pathogen occurrence in products from dairy cows.

Introduction
Management of organic waste is an important problem in cow breeding, particularly the faecal waste in a dairy farm. Composting has been recognized as an effective technique for the treatment of organic wastes (Ran et al., 2017). However, composting requires specific processes including temperature, environmental conditions and special additives to remove harmful substances in faeces, such as harmful gases, heavy metals, parasite eggs, pathogens and antibiotic resistance genes (ARGs) (Huang et al., 2017). Unfortunately, with the increase of people’s demand for dairy products and the expansion of cow breeding scale, faeces are produced faster than they can be composted. The remaining faeces are usually piled up on cow breeding farms, dried under natural conditions and then used as cow bedding material or continuously piled up without effective management (Blowey et al., 2013). In this process, the harmful ingredients, including virulence genes and ARGs of pathogens in faeces, may endanger the food safety of dairy products by integrating into the human microbiome and make humans drug-resistant (Hoelzer et al., 2017).

Pathogens and antibiotic residues are the major harmful compositions in manure (faeces accumulated under natural conditions). According to the statistics of the World Health Organization (WHO), there are approximately 1.5 billion diarrhoea patients in the world every year, and 70% of those cases were caused by food contaminated with microorganisms (Akeda, 2015). Delahoy
et al. (2018) reported that most pathogenic bacteria in food may result from the excretion of livestock and poultry faeces; the pathogens can survive in water, soil and other areas of environments for a long time and then affect human health through crops or animal products. At present, more than 150 human or animal pathogens have been identified in faeces (McDaniel et al., 2014). Thus, it is necessary to investigate the characterization of pathogenic microorganisms in fresh faeces and manure.

In China, nearly half of the antibiotics consumed (162 000 tons in 2013) are used in animal husbandry, of which substantial amounts could end up in manure (Zhang et al., 2015). The widespread usage of antibiotics in the livestock and poultry industries could lead to diseases and promote the abundances of antibiotic-resistant bacteria and ARGs increasing in the environment (Hu and Cheng, 2016). High concentrations of antibiotics and ARGs have been found in livestock faeces (Cheng et al., 2014; Karkman et al., 2019). In addition, there is a growing risk of ARGs spreading to pathogens via horizontal gene transfer (HGT), which could make antibiotics vestigial even if the bacteria carrying ARGs die (Wintersdorff et al., 2016), as some ARGs may persist even when the antibiotic pressure is gone (Zhao et al., 2017; Zhang et al., 2018). The hazards of piled up cow manure have received less attention, despite the fact that it is widely used for crop or vegetables production in China. Although dairy cows often have a lower antibiotic usage intensity than other meat-producing animals (Wang et al., 2017), cow manure was also found to contain diverse ARGs (Noyes et al., 2016; Zhou et al., 2016). The spread of ARGs to pathogens can restrict, the therapeutic potential of antibiotics, thereby posing a potential threat to the health of humans and livestock. However, little information is available with respect to the levels and changes in pathogenic bacteria and ARGs when faeces accumulate to manure in dairy cow farms.

In the present study, we provide insights into the variations in the microbial community, pathogens and ARGs when fresh faeces accumulate to manure. The accumulation of manure in dairy farms without effective manure management would lead to the enrichment of pathogenic bacteria and ARGs, increase the risk of diseases transmitted by faecal contamination, and may threaten the health of dairy cows and the safety of dairy products.

Results

Findings from the metagenomic raw data

Metagenomic raw data (mean ± SEM, raw reads, group F: 1.174E + 8 ± 0.957E + 7, group M: 1.783E + 8 ± 0.541E + 8) were filtered to clean data (mean ± SEM, clean reads, group F: 1.171E + 8 ± 0.953E + 7, group M: 1.776E + 8 ± 0.541E + 8) and assembled. We obtained an average of 241.6 ± 37.0 k (mean ± SEM, k- kilo) and 308.9 ± 90.1 k (mean ± SEM, k- kilo) counts for fresh faeces and manure samples (Table S1). The Bray-Curtis distance of relative abundance (RA) suggests that there are differences and diversity between fresh faeces and manure samples. The length of distribution of cleaned reads is shown in Fig. S1 and Table S1. Taken together, these data then being used in a subsequent analysis for changes in the microbial community, pathogenicity and antibiotic resistance between fresh faeces and manure.

The diversity composition of microorganisms in faeces

A total of 1 651 686 high-quality assembled counts were generated, the counts were then aligned against available microbial genomes from the NCBI-NR database. Taxonomic composition was generated for all samples at the levels of kingdom, phylum, class, order, family, genus and species, as shown in Table S2. The results indicated that Bacteria (including Archaea, Bacteria, Eukaryota and Viruses) was predominant in fresh faeces samples (97.29%) and manure samples (97.49%) at the kingdom level.

The RAs of taxonomy were measured by assessing the relative number of species-specific reads corresponding to queried reference sequences. The majority of species-specific mapped sequence data could be attributed to a relatively small number of individual species. With the filter criterion RAs > 1%, we obtained 6 phyla, 14 classes, 21 orders, 29 families, 27 genera and 18 species in faeces samples. The RAs (%) of microbiome composition were shown in Fig. 1 and Table S3. Despite the highly diverse bacterial communities between group F and group M, the microbial compositions of the fresh faeces were distinctively different from those of manure at every taxonomic level (Fig. 1, Tables S2-S4).

When comparing the abundance of the bacterial phylum, the RAs of Proteobacteria (11.96% vs. 50.91%) and Actinobacteria (6.7% vs. 27.14%) were highly enriched in manure compared with those in fresh faeces ($P < 0.05$). In contrast, the RAs of Firmicutes (34.37% vs. 4.86%) and Bacteroidetes (44.78% vs. 15.03%) were significantly decreased in manure compared with those in fresh faeces ($P < 0.01$) (Fig. 2A, Fig. S3A). A similar trend of significant change was found at the class level and order level; four significantly changed classes and orders were subordinated to four corresponding significantly changed phyla. At the class level, Gammaproteobacteria and Actinobacteria RAs were highly enriched in manure compared with those in fresh faeces ($P < 0.05$). RAs of Clostridia and Bacteroidia were significantly decreased in manure compared with those in...
fresh faeces ($P < 0.01$) (Fig. 2B, Fig. S3B). At the order level, RAs of Xanthomonadales and Micrococcales were highly enriched in manure compared with those in fresh faeces ($P < 0.05$). RAs of Clostridiales and Bac teroidales were decreased significantly in manure compared with those in fresh faeces ($P < 0.01$) (Fig. 2C, Fig. S3C).

There were 17 statistically significant differences between the F and M groups at the family level. The RAs of 8 of them were significantly decreased in manure compared with those in fresh faeces ($P < 0.05$), while the RAs of 9 of them were highly enriched in manure compared with those in fresh faeces ($P < 0.01$) (Fig. 2C, Fig. S3C).

The microbial composition was also distinctly different at the genus level. Among the top 27 most abundant genera (RA $> 1\%$), the RAs of Lysobacter, Pseudoxanthomonas, Nocardioides, Stenotrophomonas, Xanthomonas, Streptomyces and Corynebacterium were highly enriched in manure compared with those in fresh faeces ($P < 0.05$) (Fig. 2D, Fig. S3D). The microbial composition was also distinctly different at the genus level. Among the top 27 most abundant genera (RA $> 1\%$), the RAs of Lysobacter, Pseudoxanthomonas, Nocardioides, Stenotrophomonas, Xanthomonas, Streptomyces and Corynebacterium were highly enriched in manure compared with those in fresh faeces ($P < 0.05$) (Fig. 2D, Fig. S3D). The RAs of Treponema, Clostridium, Ruminoclostridium, Alstipes, Blautia, Lachnoclostridium, Bacteroides, Eubacterium, Prevotella, Ruminococcus, Oscillibacter, Flavonifractor, Intestiminonas and Faecalibacterium were significantly decreased in manure compared with those in fresh faeces ($P < 0.05$) (Fig. 2E, Fig. S3E).

Collectively, these data indicated that there were highly diverse bacterial communities in these 6 faecal samples. Most importantly, the microbial composition was significantly changed during faecal accumulation.

The diversity of pathogenic microorganisms in fresh faeces and manure

To obtain more in-depth view of the pathogenic microbiota present within fresh faeces and manure in dairy farm, the PHI database was used to detect differentially abundant taxa of pathogenic bacteria, virulence genes and related diseases, using the default parameters. We obtained 87 pathogenic genera in fresh faeces and manure samples. Of these, 53 human and animal pathogenic genera, 29 enphytotic genera and 5 unknown pathogenic genera were identified (Fig. 3), suggesting that there were highly diverse pathogenic communities in faecal samples in dairy cow breeding farm.
After PHI database annotation, the RAs of 53 human and animal pathogenic genera were different between group F and group M (Fig. 4A, Table 1). Compared with group F, group M exhibited substantial enrichment in total FPKM reads of pathogenic genera (human and animal pathogenic and enphytotic genera) ($P < 0.01$) (Fig. 4B, Fig. S4C). With the filter criteria RAs > 0.5% and $P < 0.05$, we found the RAs of 13 human and animal pathogenic genera were significantly increased in manure compared with those in fresh faeces, namely, those of *Acinetobacter*, *Bordetella*, *Bacillus*, *Actinobacillus*, *Cryptococcus*, *Burkholderia*, *Candida*, *Aspergillus*, *Mycobacterium*, *Beauveria*, *Brucella*, *Helicobacter* and *Flavobacterium* (Fig. 4C). Meanwhile, the RAs of 6 enphytotic genera were significantly increased in manure samples under the same filter criteria (Fig. S4A,B). These data suggested that pathogenic microbes could proliferate when faeces accumulated to manure.

We analysed the RAs of pathogenic genes and virulence genes in all samples to investigate whether the pathogenic microbes were enriched after faeces accumulation. Compared with group F, group M exhibited substantial enrichment of total FPKM reads of human and animal pathogenic virulence and pathogenic genes ($P < 0.01$) (Fig. 4D). With the filter criteria RAs > 0.5%, pathogenic genes and virulence genes were related to Pathogen-genus and the results list in Table 1, a total of 307 virulence genes and 32 pathogenic genes were found to be differentially expressed between group F and group M, of which 43 virulence genes and 6 pathogenic genes were significantly differentially expressed (Fig. 4E, F, G). There were 22 pathogenic genes (51.16%) and 232 virulence genes (75.57%) upregulated in manure compared with their expression in fresh faeces, of which 3 pathogenic genes and 40 virulence genes were upregulated significantly (Fig. 4E, G). Similarly, analysis of enphytotic virulence and pathogenic genes showed the same result (Fig. S4D, F, G). These data indicated that pathogenicity and virulence genes will be enriched with the enrichment of pathogens when faeces accumulate to manure.

© 2020 The Authors. *Microbial Biotechnology* published by John Wiley & Sons Ltd and Society for Applied Microbiology. *Microbial Biotechnology*, 13, 1039–1053.
Additionally, we found that the genera (M vs. F = 52 vs. 45) and the related diseases (M vs. F = 68 vs. 57) of human and animal pathogens in manure were higher than in fresh faeces (Fig. 4H). These results suggested that the pathogens of the cow breeding farm environment could increase their harmfulness in manure through proliferating usage of fresh faeces as a medium. Moreover, the RAs of human and animal pathogens, including *Enterococcus*, *Klebsiella*, *Porphyromonas*, *Legionella*, *Salmonella*, *Vibrio*, *Yersinia*, *Escherichia*, *Edwardsiella*, *Pseudomonas* and *Streptococcus*, were enriched in manure compared with those in fresh faeces, but the P-value of these changes was not significant (Table 1). We found the FPKM of pathogens, human and animal pathogens, virulence genes and pathogenic genes enhanced in group M with increased manure stacking depth (M3-15 cm > M2-10 cm > M1-5 cm) (Fig. 4I,J). These results indicated that manure stacking depth could influence the enrichment of pathogens.

Characterization of antibiotic resistance in fresh faeces and manure

To investigate ARGs in fresh faeces and manure, assembled genes were clustered and then analysed for the abundance of ARGs, ARG subtypes and antibiotic resistance drug types between group F and group M (Table 2, Tables S5, S6). In total, 45 ARGs and 96 ARG subtypes were differentially expressed in all samples (Table S5, Fig. 5A,B). ARGs were found with higher abundance (6065.33 ± 2375.33 vs. 2566.33 ± 355.87, P < 0.05) in manure than in fresh faeces (Fig. 5C). Compared with group F, group M exhibited upregulation of 35 ARGs (77.78%) and 71 ARG subtypes (68.93%) (Table S5). These results suggested that the overall level of antibiotic resistance was enhanced in the process of faecal accumulation.

With the filter criteria fold change > 2 and P-value < 0.05, we obtained 11 ARGs and 22 ARG subtypes that were differentially expressed between group F and group M (Fig. 5D,E). The RAs of *tlrc* (mls_abc), *cara* (mls_abc), *smed* (smedef) and *vatb* (vat) were enhanced after faecal accumulation (RA > 1%, P < 0.05). Furthermore, the RAs of *vanrg* (vang), *vanug* (vang) and *tet37* (tet_flavo) were reduced during faecal accumulation (RAs > 1%, P < 0.05) (Fig. 5F, G). It is interesting that all significantly different ARGs except *smed* (smedef) were found with higher RAs in fresh faeces than in manure and then enhanced or reduced after faecal accumulation. The
increased RAs of smedef was much lower in group F (0.05 ± 0.02%) and significantly higher in group M (5.78 ± 3.19%), with its own characteristics. The results demonstrated that there was a large number of ARGs in cow faeces on the breeding farm; a fraction of ARGs decreased after faecal accumulation, but most of the ARGs increased during the faecal accumulation process, including the ones that were present in the fresh faeces and exogenous sources or the ones newly produced during faecal accumulation.

Subsequently, we annotated single antibiotic resistance (SAR) using ARGs. A total of 43 SARs were obtained with differential RAs between group F and group M (Fig. 5H). There were 25 (6/25 RAs > 1%) positive results of SAR in fresh faeces and 43 (13/43 RAs > 1%) positive results of SAR in manure (Fig. 5I). Compared with those in group F, RAs of 18 SAR were significantly increased and of 2 SAR were significantly decreased in group M (Fig. 5J). The SAR of vancomycin was with the highest number of reads and decreased level during faecal accumulation. Macrolide, lincomamide and streptogramin_b had the highest number of reads (both in group F and group M) and increased levels during faecal accumulation (Fig. 5K, Table 2). We also found 13 SARs that were much lower in group F and significantly higher in group M, such as the ARG smedef (Table 2). Multidrug antibiotic resistance analysis indicated that the type lincomamide/macrolide/
Table 1. Differential pathogenic genuera composition and related diseases in fresh faeces (F) and waste manure (M) samples.

| Pathogen-genus | Related diseases | Number of contigs (FPMK) | Fold change | P-value |
|----------------|-----------------|--------------------------|-------------|---------|
| Acinetobacter  | Nosocomial infections | 95 | 739 | 0 | 19 954 | 19 409 | 33 034 | 86.70 | 0.006 |
| Actinobacillus | Lung infections, Bacteremia | 168 | 946 | 0 | 14 311 | 17 272 | 30 593 | 55.76 | 0.015 |
| Aeromonas      | Intestinal and extraintestinal infections | 0 | 1 | 0 | 34 | 77 | 737 | 424.00 | 0.282 |
| Arthrobotsys   | Nematode trapping fungus | 0 | 238 | 0 | 511 | 387 | 610 | 6.31 | 0.014 |
| Aspergillus    | Aspergillosis, Invasive pulmonary aspergillosis | 168 | 852 | 0 | 14 728 | 16 393 | 32 402 | 62.22 | 0.021 |
| Bacillus       | Gastrointestinal diseases and local and systemic infections, Anthrax | 142 | 58 | 0 | 4466 | 26 664 | 53 557 | 48.37 | 0.018 |
| Beauveria      | White muscardine disease | 132 | 87 | 0 | 6847 | 10 346 | 32 402 | 107.90 | 0.027 |
| Bipolaris      | Spontaneous infection | 0 | 0 | 0 | 1 | 2 | 0.30 | 0.158 |
| Bordetella     | Bordetella pertussis | 36 | 46 | 0 | 3699 | 3143 | 5747 | 151.67 | 0.006 |
| Borrelia       | Lyme disease | 1 | 0 | 0 | 204 | 150 | 117 | 235.50 | 0.003 |
| Brucella       | Brucellosis | 57 | 41 | 0 | 4024 | 3045 | 8104 | 153.26 | 0.032 |
| Burkholderia   | Melioidosis, Cystic fibrosis | 288 | 1894 | 0 | 26 460 | 26 664 | 53 557 | 48.87 | 0.018 |
| Campylobacter  | Gastroenteritis | 1 | 2 | 0 | 226 | 136 | 828 | 297.50 | 0.020 |
| Candida        | Candidiasis | 268 | 476 | 0 | 8582 | 9566 | 18 647 | 49.39 | 0.020 |
| Chlamydia      | Pneumonitis; upper genital tract infections | 0 | 0 | 0 | 26 | 0 | 0 | 26.00 | 0.374 |
| Claviceps      | Ergot | 1 | 1 | 0 | 142 | 222 | 157 | 173.67 | 0.002 |
| Clostridium    | Enteric diseases | 5 | 0 | 0 | 311 | 725 | 521 | 280.67 | 0.013 |
| Coccidioides   | Respiratory disease | 0 | 0 | 0 | 62 | 37 | 69 | 168.00 | 0.004 |
| Coxiella       | Q fever | 47 | 72 | 0 | 685 | 864 | 1056 | 21.71 | 0.002 |
| Cronobacter    | Meningitis; bacteremia | 22 | 10 | 0 | 514 | 331 | 683 | 46.30 | 0.008 |
| Cryptococcus   | Cryptococcosis | 287 | 1047 | 0 | 12 838 | 15 471 | 27 663 | 41.93 | 0.016 |
| Edwardsiella   | Enteric septicaemia, Haemorrhagic septicaemia | 7612 | 10 420 | 8578 | 21 292 | 22 645 | 62 583 | 4.00 | 0.121 |
| Enterococcus   | Nosocomial infections, Endocarditis, Probiotic gut bacteria | 1111 | 1540 | 1528 | 7091 | 32 518 | 11 008 | 5.11 | 0.063 |
| Escherichia    | Infections, Avian colibacillosis | 800 | 2556 | 11 19 | 5641 | 13 363 | 31 708 | 11.33 | 0.118 |
| Flavobacterium | Bacterial cold-water disease | 129 | 154 | 219 | 2918 | 4629 | 8947 | 32.79 | 0.041 |
| Francisella    | Ferric and ferrous iron acquisition | 1465 | 2594 | 2000 | 4072 | 4448 | 11 637 | 3.33 | 0.131 |
| Haemophilus    | Glassers Disease | 147 | 1033 | 406 | 836 | 1567 | 5625 | 5.06 | 0.229 |
| Helicobacter   | Gastric infections | 248 | 693 | 378 | 1809 | 2480 | 4364 | 6.56 | 0.035 |
| Kingella       | Diseases of the skeletal system in children and infective endocarditis | 0 | 0 | 0 | 38 | 72 | 33 | 143.00 | 0.018 |
| Klebsiella     | Urinary tract infections; nosocomial pneumonia; intra-abdominal infections | 460 | 2017 | 727 | 7651 | 10 765 | 24 887 | 13.51 | 0.066 |
| Legionella     | Legionnaires disease | 436 | 830 | 687 | 2058 | 3090 | 7105 | 6.27 | 0.090 |
| Leishmania     | Leishmaniasis | 0 | 1 | 1 | 13 | 18 | 10.67 | 0.119 |
| Leptospirosis  | Leptospirosis | 246 | 286 | 108 | 23 | 178 | 141 | 0.53 | 0.236 |
| Listeria       | Listeriosis | 3416 | 5173 | 4550 | 6293 | 2990 | 7756 | 1.30 | 0.435 |
| Mycobacterium  | Tuberculosis | 215 | 242 | 346 | 19 184 | 8354 | 24 164 | 64.31 | 0.022 |
| Mycoplasma     | Contagious agalactia | 133 | 127 | 161 | 21 | 41 | 67 | 0.31 | 0.005 |
| Neisseria      | Neisseria meningitidis | 4 | 5 | 6 | 170 | 30 | 565 | 64.75 | 0.043 |
| Paenibacillus  | American foulbrood | 0 | 0 | 0 | 181 | 141 | 72 | 394.00 | 0.015 |
| Photobacterium | Immunosuppression; septicaemia; subsequent death | 0 | 0 | 0 | 0 | 1 | 0 | 1.00 | 0.374 |
| Porphyromonas  | Periodontitis | 7407 | 9962 | 7922 | 16 788 | 15 272 | 30 820 | 2.49 | 0.067 |
| Proteus        | Urinary tract infections | 101 | 729 | 185 | 439 | 877 | 2272 | 3.53 | 0.218 |
| Pseudomonas     | Septicaemia, Infections | 3376 | 4803 | 4589 | 21 262 | 43 872 | 12 5078 | 14.90 | 0.134 |

© 2020 The Authors. Microbial Biotechnology published by John Wiley & Sons Ltd and Society for Applied Microbiology, Microbial Biotechnology, 13, 1039–1053

Microbial community and risk shifts in stored manure
streptogramin_b had the highest increased level during faecal accumulation (Table S5-S6). These data indicated that the process of faecal accumulation could result in significantly increased microbial antibiotic resistance.

Interestingly, we also found FPKM of ARGs enhanced in group M with increased manure stacking depth (M3-15 cm > M2-10 cm > M1-5 cm, Fig. 5L), similar to the result for pathogens in group M (Fig. 4I,J). The results lend further credence to the hypothesis that manure stacking depth could influence the enrichment of antibiotic resistance.

Discussion

Microbiome studies described the significance of the microbial community that was associated with the host organism (Brooks et al., 2017). However, less than 1% of all microbial species can be cultured in vivo (Locey and Lennon, 2016). Metagenomics, which a method could directly analyses the total DNA from environmental samples, provides a powerful strategy for unveiling novel microbes in microbial communities without the technical challenges of cultivation (Doane et al., 2017). In this study, we analysed the metagenomic data from three fresh faeces and three manure samples in a dairy cow breeding farm in Ningxia. Ningxia is one of the top ten pastoral areas in China. We found highly diverse and distinctly different bacterial communities between fresh faeces and manure. Present studies agree that microbial diversity is a major feature in fresh faeces or composted samples (Kim et al., 2018). We and others found that the dominant phyla of the community structure

| Pathogen-genus | Related diseases | Number of contigs(FPKM) | Fold change | P-value |
|----------------|------------------|-------------------------|-------------|---------|
| Yersinia       | Gut-associated diseases, Pneumonic plague | 697 1103 997 2678 4604 11 399 | 6.68 | 0.116 |
| Cercospora     | unknown | 3 1 0 15 3 8 | 5.20 | 0.111 |
| Citrobacter    | unknown | 1 1 0 233 90 127 | 150.00 | 0.025 |
| Metarhizium    | unknown | 15 8 10 196 909 142 | 36.68 | 0.177 |
| Saccharomyces  | unknown | 1819 2771 2211 1960 3812 10 976 | 2.46 | 0.296 |
| Xenorhabdus    | unknown | 70 332 88 524 1204 3986 | 11.64 | 0.177 |

Table 1. (Continued)
studies (Kim et al., 2018; Muñoz-Vargas et al., 2018). Previous research has shown that the largest number of pathogen genes was detected in dairy waste and they may come from farm environments or other wastes, which was a reminder of the potential risk to human health presented by the farm environment and consumption of unpasteurized milk (Sledz et al., 2017). We found the species and RAs of human and animal pathogens were significantly increased during faecal accumulation. There were 52 human and animal pathogenic genera in manure compared with 45 kinds in fresh faeces. Of these, the RAs of 13 human and animal pathogenic genera were significantly increased in manure compared with those in fresh faeces (Fig. 4C). The results indicated that faecal accumulation could be regarded as a process of the enrichment of pathogens in faeces and the environment during the use of manure as the medium. It was likely resulted from that microbial pathogens could benefited from the rich source of available nutrients in organic manures like nitrogen, phosphorus, potassium, sodium, copper, zinc, calcium, selenium, manganese, magnesium and sulphur. (Sledz et al., 2017). At the same time, recent research indicated that stream water and rain in dairy breeding farms could help manure mix with water and expand the spread of pathogens (Haack et al., 2016). Therefore, faecal accumulation is the one of the major sources of pathogens in dairy farm environments. We analysed the human and animal pathogenic pathogens only at the genus level, despite the PHI database annotation results at the species level. These results have been sufficient to offer the potential to address both the source of and risks associated with faecal pollution, for example, the potential pathogenicity of Actinobacter in nosocomial infections (Almasaudi, 2018), Bacillus in anthrax (Welkos et al., 2015) and Actinobacillus in bacteremia (Cosford, 2018). Additionally, we found that the RAs of 6 enphytotic genera were also significantly increased in manure samples, namely, Fusarium (Fusarium ear blight, Fusarium head blight), Xanthomonas (bacterial leaf blight), Parastagonospora (glume blotch), Agrobacterium (crown gall disease), Alternaria (brown spot disease) and Ralstonia (bacterial wilt). This result suggests that pathogens from manure could spread to the plant environment and enphytotic pathogens could affect the yield and quality of crops when manure is used as a fertilizer. Thus, complex environments enhance bacterial community enphytotic pathogens could affect the yield and quality of crops when manure is used as a fertilizer. Thus, complex environments enhance bacterial community
interactions and metabolism; These results being consistent with previous studies (Häger et al., 2016; Sledz et al., 2017).

Pathogens always harm the host through virulence genes. In this study, we found that three pathogenic genes and 40 virulence genes from human and animal pathogenic genera were significantly upregulated in manure compared with their expression in fresh faeces. Among them, the top 10 virulence genes and three pathogenic genes included the USX1, MET3, URE1 and Tco1 genes produced from Cryptococcus neoformans, which may cause cryptococcosis disease in either humans or animals (Moyrand et al., 2002; Pascon et al., 2004; Feder et al., 2015; Kong et al., 2017); the bpdA and CspA genes producing from Brucella melitensis may cause ovine brucellosis in animals (Zhang, 2018); the nhaA and CheA genes produced by E. coli, which may cause meningitis or infect humans or animals (Tavaddod and Naderi-Manesh, 2016); the Fdh3 gene produced by Candida albicans, which may cause disseminated candidiasis in either humans or animals (Tillmann et al., 2015); the oqxB gene produced by Klebsiella pneumoniae, which may cause pneumonia disease in animals (Nicolas-Chanoine et al., 2018); the VC1295 gene produced by Vibrio cholerae, which may cause cholera disease in either humans or animals (Conner et al., 2017); the RV3232c gene associated with Mycobacterium tuberculosis, which may cause tuberculosis in either humans or animals (Singh et al., 2016); and the PA2414 gene associated with Pseudomonas aeruginosa which

Fig. 5. RA characterization of antibiotic resistance genes (ARGs) and types in fresh faeces and manure samples. (A&B) Heat map analysis of differential abundances of ARGs and ARG subtypes. (C) Total FPKM of ARGs between group F and group M. (D&E) Volcano plot analysis of the difference in ARGs and ARG subtypes between group F and group M. (F&G) Column chart analysis of significantly different FPKM of ARGs and ARG subtypes between group F and group M. (H&J) Heat map analysis of differential RAs of single antibiotic resistance (SAR). (I) Number of SAR-positive results and significant positive results (RAs>1%) between group F and group M. (K) Volcano plot analysis of the differential abundance of an SAR between group F and group M. (L) FPKM of ARGs enhanced in group M with increased manure stacking depth (M1-5 cm, M2-10 cm, M3-15 cm). *,+ = P < 0.05, **,++ = P < 0.01.
may cause nosocomial infections disease in either humans or animals (Dubern \textit{et al.}, 2015). The results for the virulence genes were consistent with those for pathogenic bacteria. Interestingly, we found FPKM of pathogens, human and animal pathogens, virulence genes and pathogenic genes enhanced in group M with increased manure stacking depth (M3-15 cm > M2-10 cm > M1-5 cm), which made some human and animal pathogens have higher RAs and be enriched in manure compared with those in fresh faeces, but the P-value of these changes was not significant. Such species included \textit{Pseudomonas}, \textit{Streptococcus} and \textit{Escherichia}, which may infect either humans or animals. We believe that these pathogens are noteworthy and need to be analysed in a subsequent validation utilizing a larger cohort.

Overuse of antibiotics in animal husbandry is a worldwide problem (Ferri \textit{et al.}, 2017). China is one of the largest consumers of antibiotics worldwide (Zhang \textit{et al.}, 2015). In the present study, we found that the overall level of RAs of ARGs and ARG subtypes was increased during manure accumulation. In total, 11 ARGs and 22 ARG subtypes were enhanced in manure. Among them, \textit{tlc} (\textit{mls} \textit{abc}), \textit{cara} (\textit{mls} \textit{abc}), and \textit{vat}\textit{b} (\textit{vat}) were enhanced during faecal accumulation, with increased RAs in either fresh faeces or manure, while \textit{vanrg} (\textit{vang}), \textit{vanug} (\textit{vang}) and \textit{tet37} (\textit{tet flavo}) were reduced in the process, with higher RAs in either fresh faeces or manure. This result means that the resistances to streptogramin\textit{a} (\textit{vat}), lincosamide/macrolide/streptogramin\textit{b} (\textit{mls} \textit{abc}), vancomycin (\textit{vang}) and tetracycline (\textit{tet flavo}) were found with high RAs in fresh faeces. Previous studies reported that lincosamide/macrolide/streptogramin\textit{b} was commonly used in livestock breeding as feed additives to promote animal growth and maturity (Hao \textit{et al.}, 2015). In addition, vancomycin and tetracycline are commonly used in bacteriostasis and sterilization in dairy breeding farms (Holmes \textit{et al.}, 2018). Consistent with relevant studies, ARGs for tetracyclines were found in the manure of chickens, pigs and cows (Mitchell \textit{et al.}, 2015). Previous study indicated that tetracyclines, vancomycin and streptogramin\textit{b} should decrease in vivo after feeding of livestock or in vitro during faeces composting (Selvam \textit{et al.}, 2013; Mitchell \textit{et al.}, 2015). However, we found degradation of only vancomycin, with no change in tetracycline, but increased levels of lincosamide, macrolide, streptogramin\textit{b} and streptogramin\textit{a}. Moreover, fluoroquinolone resistance (\textit{smedef} gene) was not found in fresh faeces but increased significantly in manure, speculating that the accumulated manure was also the transfer station of ARGs to enrich the ARGs in the environment. One of the primary causes of enrichment of ARGs is HGT which could make ARGs vestigial even if the bacteria carrying ARGs die (Wang \textit{et al.}, 2018). We observed RAs of microbes and pathogenic microbe enrichment when faeces accumulated to manure. With HGT, ARGs transferred from the host bacteria to other bacteria, pathogenic microbes, even host and the environment. Eventually, humans could become the ultimate receptors of ARGs. Previous studies have also confirmed that HGT of ARGs could impact food safety by the enrichment of ARGs in the pasture or in the composting process of pig, cow and poultry manure (Soucy \textit{et al.}, 2015; Baker \textit{et al.}, 2016; Pornsukarom and Thakur, 2017).

Interestingly, consistent with the results of virulence genes and pathogens being enhanced in group M with increased manure stacking depth (M3-15 cm > M2-10 cm > M1-5 cm), a similar trend was found in ARGs in manure. It is not clear whether the changes in waste manure with stacking depth were caused by the time, local environment, or microbial interactions during accumulation. We will pay more attention to these questions by an in-depth examination.

In summary, through metagenomic analysis of fresh faeces and manure samples in a dairy farm in Ningxia, we provided evidence that (i) manure accumulation could significantly increase the abundances of the microbial and pathogenic constituents (ii) levels of virulence genes and ARGs increased with the enrichment of microbes and pathogens when faeces accumulated to manure and (iii) manure accumulation and usage without effective manure management is an unreasonable approach that could enrich pathogenic microorganisms and ARGs in the environment. Our findings provide insights into the influence of animal agriculture on manure accumulation and pollution in the breeding farm environment. Additional research of this type and a scale up would lend improved insight into the overall influence and interaction of animal waste on water, soil and other areas impacted by faecal accumulation and the factors that influence pathogen occurrence in products from dairy cows.

**Experimental procedures**

**Specimens**

A total of three fresh faeces and three manure specimens were collected in a scaled dairy cow breeding farm (Breeding stock:1500) in Ningxia, China (N38°20', E106°16'). Each fresh faecal sample was a mixture from randomly chosen fresh faeces of 10 cows. A total of three fresh faecal samples were collected from different sectors in the same dairy cow farm in May 2017. Fresh faeces from 30 cows were collected to make the three fresh faecal samples. Manure sample was a mixture of the manure randomly collected from piled up manure; the specimens were mixtures collected at three different sectors in the same dairy cow farm in May 2017.
depths (5, 10 and 15 cm), and each was mixed in six sites in manure piled area (the faeces from all dairy cows in the breeding farm) after manure piled up for about 2 months in July 2017. The specimens were sealed in a 50 ml sterilized tube and then directly transported at 4°C to the Key Laboratory of the Ministry of Education for Protection and Utilization of Special Biological Resources in western China, within 4 h. The specimens were stored in −80°C for further use. The study protocol was reviewed and approved by the ethics committees of Ningxia University (Ningxia, China).

**DNA extraction and metagenome sequencing**

DNA was extracted from 200 mg of samples with a QiAamp DNA Stool mini kit (QIAGEN, Dusseldorf, Germany) following the manufacturer’s instructions. DNA samples were quantified using NanoDrop 2000 (Thermo Scientific, Waltham, MA, USA) and confirmed using 0.8% agarose gel electrophoresis. Extracted DNA from each sample was stored at −20°C until use. The qualified DNA samples were broken into DNA fragments with a length of approximately 350 base pairs. After end repair, sequencing adaptor addition, purification and other steps, library construction from the samples were achieved using a TruSeq® Nano DNA LT Sample Prep Kit (Illumina, San Diego, CA, USA). The libraries had an insert size of approximately 350 base pairs for every sample. Each library was sequenced by Illumina HiSeq 2000 equipment (Illumina). Metagenome sequencing were used (i) to determine the microbial diversity composition in fresh faeces and manure; (ii) to determine the diversity in the pathogenic community and antibiotic resistance during manure accumulation; and (iii) to evaluate the pathogen risk of natural manure accumulation and usage without effective manure management.

**Metagenome assembly and species annotation**

An overview of the workflow developed with the tools applied at each step were list in Fig. S1. Raw sequencing reads were processed to obtain quality-filtered reads for further analysis. First, sequencing adaptors were removed from sequencing reads using Cutadapt (Grasemann, 2019). Second, low-quality reads were trimmed by using a sliding-window algorithm (Wang et al., 2015). Third, reads were aligned to the host genome using BWA (http://bio-bwa.sourceforge.net/) to remove host contamination (Li and Durbin, 2009). The following criteria were used for quality control: (i) reads were removed if they contained more than 3 N bases or more than 50 bases with low quality (<Q20), and (ii) reads were trimmed at the end with low quality (<Q20) or assigned as N. Once quality-filtered reads were obtained, they were assembled to construct a metagenome for each sample by SOAP2 software with the De Bruijn graph and overlap-layout-consensus (OLC) methods (Ye et al., 2016; Stewart et al., 2018). Contig were classified using k-mers and coverage. Gene abundance in each sample was estimated by soap.coverage (http://soap.genomics.org.cn/) based on fragments per kilobase per million mapped reads (FPKM) (Koch et al., 2018). Taxonomy of every contig (gene annotation and amino acid annotation) was obtained by aligning them against the National Center for Biotechnology Information-NR database (bacteria, fungi, archaea and viruses, e value ≤ 1E-5). Taxonomic annotation (kingdom, phylum, class, order, family, genus and species) in each sample was estimated by taxonomy annotation results, and every contig was considered an organism. The RAs of a species in a sample were denoted as the sum of the genetic abundance of that species.

**Pathogenicity and antibiotic resistance analysis**

The functional profiles of the nonredundant genes were obtained by annotation against the Gene Ontology (GO) and Kyoto Encyclopaedia of Genes and Genomes (KEGG) databases by using the DIAMOND alignment algorithm (Huson et al., 2017). Based on the taxonomic and functional profiles of nonredundant genes, the Pathogen Host Interactions database (PHI-base, http://www.phi-base.org/) was utilized to detect differentially abundant taxa (default parameters) of pathogenic bacteria, virulence genes and related diseases in every sample (Urban et al., 2017). The Antibiotic Resistance Genes Database (ARDB) was used to detect differentially abundant taxa with ARGs and antibiotic-resistant types in every sample, using the default parameters (Yang et al., 2016). Gene abundance in each sample was estimated by soap.coverage based on FPKM. Species variation and functional gene variation in microbial communities across samples was analysed using Bray–Curtis distance metric principal coordinates analysis (PCoA). A heat map was analysed using Cluster 3.0 software. A volcano plot was analysed using ggPlot2 software (R).

**Statistical analysis**

The abundances of assembled contigs were calculated based on the number of FPKM by using SPSS23.0 software (SPSS Inc. IBM, Chicago, IL, USA) and GraphPad Prism version 8.0.1 (GraphPad Software, San Diego, CA, USA). The data were presented as the mean ± Standard error (SEM) unless otherwise stated. Continuous variables were tested using a t-test. All P-values were two-tailed, and the differences were considered significant if P < 0.05* or P < 0.01**.
Microbial community and risk shifts in stored manure 1051

Acknowledgements

The study was funded by Key Technologies Research and Development Program of Ningxia (2015BZ02); Key Project of Research and Development of Ningxia Hui Autonomous Region of China (2017BN04); The Major Innovation Projects for building first-class Universities in China’s Western Region (ZKZD2017001); The project for the cultivation of scientific and innovative talent (KJT2017002). No one contributed towards the article who does not meet the criteria for authorship including anyone who provided professional writing services or materials in the study. We also thank for the efforts of all authors.

Conflict of interest

This work does not have any relationships with business-related issues, and no conflicts of interest exist in the submission of this manuscript.

Ethical approval

The study protocol was reviewed and approved by ethics committees of Ning Xia University (Ning Xia, China). The manuscripts reporting studies involving none of human participants, human data or human tissue.

Consent for publication

I would like to declare on behalf of my co-authors that the work described was original research that has not been published previously, and not under consideration for publication elsewhere, in whole or in part. The manuscript is approved by all authors for publication.

Author contribution

Guangcun DENG: designed project, revised article and coordinated all aspects of work; Xiaoling WU: designed project, revised article, technical guidance; Xu ZHANG: participated in all experiment, data analysis, create figures, wrote article; Chenjie MA: NCBI-NR database and taxonomic annotation, create tables; Wen ZHANG: PHI database analysis, data Cleansing; Wu LI: ARDB database analysis, data Cleansing; Jialin YU: specimens collected, sample preparation, documentation; Di XUE: Metagenome sequencing and analysing technical guidance, data quality control.

References

Akeda, Y. (2015) Food safety and infectious diseases. J Nutrit Sci Vitaminol 61(Suppl): S95.

© 2020 The Authors. Microbial Biotechnology published by John Wiley & Sons Ltd and Society for Applied Microbiology, Microbial Biotechnology, 13, 1039–1053

Almasaudi, S.B. (2018) Acinetobacter spp. as nosocomial pathogens: epidemiology and resistance features. Saudi J Biol Sci 25: 586–596.

Baker, M., Hobman, J. L., Dodd, C.E., Ramsden, S.J., and Stekel, D.J. (2016) Mathematical modelling of antimicrobial resistance in agricultural waste highlights importance of gene transfer rate. FEMS Microbiol Ecol 92: fiw040.

Blowey, R., Wooky, J., Russell, L., and Goss, R. (2013) Dried manure solids as a bedding material for dairy cows. Vet Rec 173: 99–100.

Brooks, A.W., Kohl, K.D., Brucker, R.M., van Opstal, E.J., and Bordenstein, S.R. (2017) Phyllosymbiosis: relationships and functional effects of microbial communities across host evolutionary history. Plos Biol 15: e1002587.

Cheng, G., Hao, H., Xie, S., Wang, X., Dai, M., Huang, L., and Yuan, Z. (2014) Antibiotic alternatives: the substitution of antibiotics in animal husbandry? Front Microbiol 5: 217.

Conner, J.G., Zamorano-Sánchez, D., Park, J.H., Sondermann, H., and Yildiz, F.H. (2017) The ins and outs of cyclic di-GMP signaling in Vibrio cholerae. Curr Opin Microbiol 36: 20–29.

Cosford, K.L. (2018) Brucella canis: an update on research and clinical management. Can Vet J-revue Veterinaire Canadienne 59: 74–81.

Delahoy, M.J., Wodnik, B., M'callelin, L., Penakalapati, G., Swarthout, J., Freeman, M.C., and Levy, K. (2018) Pathogens transmitted in animal feces in low- and middle-income countries. Int J Hygiene Environ Health 221: 661–676.

Doane, M.P., Haggerty, J.M., Kacev, D., Papudeshi, B., and Dinsdale, E.A. (2017) The skin microbiome of the Common thresher shark (Alopias vulpinus) has low taxonomic and potential metabolic β-diversity. Environ Microbiol Rep 9: 357–373.

Dubern, J.F., Cigana, C., De Simone, M., Lazenby, J., Juhas, M., Schwager, S., et al. (2015) Integrated whole-genome screening for Pseudomonas aeruginosa virulence genes using multiple disease models reveals that pathogenicity is host specific. Environ Microbiol 17: 4379–4393.

Feder, V., Knettisch, L., Staats, C.C., Vidal-Figueiredo, N., Ligabue-Braun, R., Carlini, C.R., and Vainstein, M.H. (2015) Cryptococcus gattii urease as a virulence factor and the relevance of enzymatic activity in cryptococcosis pathogenesis. FEBS J 282: 1406–1418.

Ferri, M., Ranucci, E., Romagnoli, P., and Giaccone, V. (2017) Antimicrobial resistance: a global emerging threat to public health systems. Crit Rev Food Sci Nutrit 57: 2857–2876.

Grassmann, F. (2019) Conduct and quality control of differential gene expression analysis using high-throughput transcriptome sequencing (RNAsSeq). Methods Mol Biol 1834: 29–43.

Häger, A.C., Mayo, M., Price, E.P., Theobald, V., Harrington, G., Machunter, B., et al. (2016) The melioidosis agent Burkholderia pseudomallei and related opportunistic pathogens detected in faecal matter of wildlife and livestock in northern Australia. Epidemiol Infect 144: 1924–1932.

Haack, S.K., Duris, J.W., Kolpin, D.W., Focazio, M.J., Meyer, M.T., Johnson, H.E., et al. (2016) Contamination
with bacterial zoonotic pathogen genes in U.S. streams influenced by varying types of animal agriculture. Sci Total Environ 563–564: 340–350.

Hao, R., Zhao, R., Qiu, S., Wang, L., and Song, H. (2015) Antibiotics crisis in China. Science 348: 1100–1101.

Hoelzer, K., Wong, N., Thomas, J., Talkington, K., Jungman, E., and Coukell, A. (2017) Antimicrobial drug use in food-producing animals and associated human health risks: what, and how strong, is the evidence? BMC Vet Res 13: 211.

Holmes, A., Holmes, M., Gottlieb, T., Price, L.B., and Sundsfjord, A. (2018) End non-essential use of antimicrobials in livestock. BMJ 360: k259.

Hu, Y., and Cheng, H. (2016) Health risk from veterinary antimicrobial use in China’s food animal production and its reduction. Environ Pollut 219: 993–997.

Huang, J., Yu, Z., Gao, H., Yan, X., Chang, J., Wang, C., et al. (2017) Chemical structures and characteristics of animal manures and composts during composting and assessment of maturity indices. Plos One 12: e0178110.

Huson, D.H., Tappu, R., Bazinet, A.L., Xie, C., Cummings, M.P., Nieselt, K., and Williams, R. (2017) Fast and simple protein-alignment-guided assembly of orthologous gene families from microbiome sequencing reads. Microbiome 5: 11.

Karkman, A., Pärnänen, K., and Larsson, D. J. (2019) Fecal pollution explains antibiotic resistance gene abundances in anthropogenically impacted environments. Nat Commun 10: 80.

Kim, M., Yun, J.I., Won, S.G., and Park, K.H. (2018) Changes of microbial diversity during swine manure treatment process. Polish J Microbiol 67: 109–112.

Koch, C.M., Chiu, S.F., Akbarpour, M., Bharat, A., Ridge, K.M., Bartom, E.T., and Winter, D.R. (2018) A beginner’s guide to analysis of RNA-seq data. Am J Respir Cell Mol Biol 59: 145–157.

Kong, Q., Yang, R., Wang, Z., Zhou, W., Du, X., Huang, S., et al. (2017) Transcriptomic and virulence factors analyses of Cryptococcus neoformans hyphoxia response. Apmls 125: 236–248.

Li, H., and Durbin, R. (2009) Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics 25: 1754–1760.

Locey, K.J., and Lennon, J.T. (2016) Scaling laws predict global microbial diversity. Proc Acad Nat Sci USA 113: 5970–5975.

Mcdaniel, C.J., Cardwell, D.M. Jr, Moeller, R.B., and Gray, G.C. (2014) Humans and cattle: a review of bovine zoonoses. Vector Borne Zoonotic Dis 14: 1–19.

Mitchell, S., Ullman, J., Bary, A., Cogger, C., Teel, A., and Watts, R. (2015) Antibiotic degradation during thermophilic composting. Water Air Soil Pollut 226: 13.

Moyerand, F., Klaproth, B., Himmelreich, U., Dromer, F., and Janbon, G. (2002) Isolation and characterization of capsule structure mutant strains of Cryptococcus neoformans. Mol Microbiol 45: 837–849.

Muñoz-Vargas, L., Opiyo, S.O., Digianantonio, R., Williams, M.L., Wijeratne, A., and Habing, G. (2018) Fecal microbiome of periparturient dairy cattle and associations with the onset of Salmonella shedding. Plos One 13: e0196171.
Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.