A bacterial genome and culture collection of gut microbial in weanling piglet

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49 ABSTRACT

50 The microbiota hosted in the pig gastrointestinal tract are important for
 51 productivity of livestock. However, the individual species and functional
 52 repertoires that make up the pig gut microbiome remain largely undefined. Here
 53 we comprehensively investigated the genomes and functions of the piglet gut
 54 microbiome using culture-based and metagenomics approaches. A collection
 55 included 266 cultured genomes and 482 metagenome-assembled genomes
 56 (MAGs) that were clustered to 428 species across 10 phyla was established.
 57 Among these clustered species, 333 genomes represent potential new species.
 58 Less matches between cultured genomes and MAGs revealed a substantial bias
 59 for the acquisition of reference genomes by the two strategies. Glycoside
 60 hydrolases was the dominant category of carbohydrate-active enzymes. 445
 61 secondary metabolite biosynthetic genes were predicted from 292 genomes with
 62 bacteriocin being the most. Pan genome analysis of *Limosilactobacillus reuteri*
 63 uncover the biosynthesis of reuterin was strain-specific and the production was
 64 experimentally determined. These genomic resources will enable a
 65 comprehensive characterization of the microbiome composition and function of
 66 pig gut.

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68 INTRODUCTION

69 Pigs are economically important livestock, widely used as monogastric animal
 70 model for enteric microbiological studies, and serve as most consumed meat for
 71 human worldwide^{1,2}. A large amount of microorganisms harbored in the gut of pig.
 72 The research of gut microbiome of pig has increasingly advanced our understanding
 73 of the role of the microbiota in feed conversion efficiency, pig health, and
 74 production in recent years^{3,4}.

75 Metagenomic studies have uncovered the genes, species, and functional diversity of
 76 bacteria in mammalian intestines with the advantage in avoiding the time-consuming
 77 of traditional culture-based approach and the inaccuracy of 16S rRNA sequences
 78 analysis. A gene catalog consisting of 7.7 million non-redundant genes has been
 79 constructed that draw a basic function map of the pig gut microbiota⁵. PIGC, the pig
 80 integrated gene catalog, a recent expanded gene catalog comprised 17 million
 81 complete genes that provide an expanded resource for pig gut microbiome research⁶.
 82 Metagenomic binning is an essential tool for acquiring reference genomes readily,
 83 particularly for uncultured microbiota, that has been widely used in study of human
 84 gut microbiota, but rarely applied for pigs. However, misassemblies and chimeric
 85 contigs from MAGs result in substantial biases for the analysis of the gut
 86 microbiome^{7, 8}. These metagenomic studies have greatly increased our
 87 understanding of pig gut microbiome^{6, 9}. Nevertheless, the lack of bacterial isolates
 88 and high-quality genomes limited our comprehensively understanding of the
 89 structure and function of the gut microbiota of pig and the development of probiotics
 90 for pig farming.

91 The culture-based approach has been well used for establishment of bacterial
 92 collection for the human gut microbiota¹⁰. Over 1,500 species have been
 93 successfully isolated from the human gut by using culturomics, introduced by Lagier
 94 *et al.* and 247 new species have been unveiled¹¹. Culture-based studies enable the
 95 acquisition of both live bacteria and high-quality reference genomes and provide
 96 experimental access for function exploration and intervention trials with probiotics.
 97 PiBAC, a pig intestinal bacterial collection, was constructed by cultivation of 110
 98 species and described 38 novel species from the gut of 19 pigs in Germany, United
 99 States, and Canada¹². This study highlighted the importance and necessity of
 100 continuously isolating and characterizing microbial taxa from pig gut.

101 Here we performed a study of bacterial cultivation and genome sequencing along with
 102 deep metagenome sequencing of 14 samples, including ileum and colon contents, and
 103 faeces, from weanling piglets. A total of 266 isolated bacterial genomes and 482

MAGs were obtained and investigated their functional repertoires. This study provides a comprehensive view of the microbiome composition and the function landscape of the gut of weanling piglets and a valuable bacterial resource for further experimentations.

RESULTS

The metagenome sequencing described community composition of microbiota in different gut regions of weanling piglets

A total of 42 samples, including 15 ileum contents, 18 colon contents and 9 faeces, were collected from 42 weanling piglets from Guangdong Academy of Agricultural Sciences. Every 3 samples from one line were combined into one sample which ultimately resulted in 14 samples. To investigate the microbiota composition in each sample, we first performed deep metagenomic sequencing for these samples and generated 20 Gb of data for each sample on average. Notably, over 12% of reads mapped to bacteria that could not be classified at the species level, which represent novel species in the gut of weanling piglets (Supplementary Fig. 1). 99.8% of the bacterial reads were assigned into 10 phyla. The phylum Firmicutes dominated the gut microbiota in the ileum, colon, and faeces, accounting for 98.9%, 67%, and 76.8% of the bacterial sequences, respectively (Figs. 1a, b). We also discovered *Lactobacillus* were common present in 3 regions and accounted for the highest proportion in samples from faeces and ileum and some samples from colon (Supplementary Fig. 2a, b). The higher microbial diversity, including the Shannon index, was observed in the colon as compared with the faeces and ileum (Fig. 1c), consistent with previous studies^{13, 14}. The principal coordinates analysis showed that the microbiota composition of the faeces and colon were similar and were different from that of the ileum (Fig. 1d). These results indicated the regional similarities and differences in microbiota composition and the beneficial bacteria, like *Lactobacillus* dominated the gut microbiota of weanling piglets, were valuable resource for the

132 furtherly cultured-based study.

133 **Bacteria culturing and genome sequencing of gut of weanling piglets**

134 To advance our understanding of the diversity of gut microbiota and acquire bacterial
135 isolates from the weanling pig gut, we used a variety of culture methods, including
136 nutrient-rich medium (MPYG, with or without sheep blood), oligotrophic medium
137 (R2A), Columbia Agar, spore medium, *etc.* In total, 25 culture conditions were used
138 (Supplementary method). Most of the bacteria were cultured in sheep blood-MPYG,
139 MPYG, and sheep blood-spore medium. A total of 1,476 strains were obtained and
140 identified using 16S rRNA gene sequence, the detail information is provided in
141 Supplementary Table 2. These isolates belonged to 5 different phyla, including
142 Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, and Fusobacteria
143 (Supplementary Fig. 3). *Limosilactobacillus reuteri* was the most abundant, which
144 represented 18% of the taxa isolated and half of these 1,476 bacteria were
145 *Lactobacillus* (recently divided as *Lactobacillus*, *Ligilactobacillus*, and
146 *Limosilactobacillus*), representing the beneficial bacteria in gut of piglets
147 (Supplementary Fig. 4). Most of these bacteria were the first time isolated from the
148 pig gut microbiota, which expanded the bacteria diversity of the gut microbiota of pig.
149 Notably, the isolated bacteria from the ileum, colon, and faeces were much different
150 with highest species diversity of bacteria from faeces sample, that consistent with the
151 analysis of metagenome (Supplementary Fig. 4). All bacteria have been deposited in
152 the China National Genebank (CNGB) Shenzhen for public accessibility.

153 **Comparison of cultured genomes and MAGs**

154 We selected 266 representative strains that covered the bacterial diversity of the 1,476
155 isolates for genome sequencing. After assembly, 266 high-quality genomes with
156 completeness more than 90% and contamination less than 5% were obtained
157 (Supplementary Table 3a), of which 195 genomes (73.31%) were more than 99%
158 completeness (Supplementary Fig. 5a), indicating that the great majority of the
159 genomes were relatively complete. Based on the 95% average nucleotide identity

(ANI), we clustered the 266 genomes into 59 species-level clusters. The vast majority of clusters were Firmicutes (242 genomes, 52 clusters), followed by Proteobacteria (18 genomes, 3 clusters), Actinobacteriota (4 genomes, 2 clusters), Bacteroidota (1 genome, 1 cluster) and Fusobacteriota (1 genome, 1 cluster) according to the taxonomic annotation of these genomes (Fig. 2a). Notably, 10 out of 59 clusters (23 genomes) lacked a species-level match with the GTDB-Tk database (Fig. 2a and Supplementary Table 3a).

By Assembling and binning metagenomic sequence data from 14 samples, we reconstructed 482 non-redundant MAGs with completeness more than 70% and contamination less than 5% (Supplementary Table 3b). Only 223 (46.27%) MAGs were high-quality genomes (completeness more than 90%), 11 of which with completeness more than 99% (Supplementary Fig. 5b), which indicated that the quality of the genomes generated by culture-based methods was generally higher than metagenomic assembly. To evaluate the species bias caused by the two methods, we clustered 489 high-quality genomes. The results showed that MAGs and isolated genomes had 74.63% and 25.42% of unique species-clusters, respectively (Supplementary Fig. 6a, b), illustrating that the combination of two methods is needed to more comprehensively represent the species of the pig gut microbiota. We thus clustered the 748 genomes into 428 species-clusters to integrate the reference genomes. According to the GTDB classification annotations, 5 clusters (5 MAGs) were Archaea, and the remaining clusters were bacteria from 10 phyla (Fig. 2a and Supplementary Table 4). It is worth noting that 77.80% of clusters (333 clusters) could not be matched with any existing species, which represent novel species, and 96.70% (322 clusters) of the novel species without isolate genome representative in this study (Fig. 2a).

Previous studies have used metagenomics or culture-based method to study the intestinal microbes of pigs. Wang *et al.*⁹ generated 360 high-quality assembled genomes for pig fecal microbiome. And Wylensek *et al.*¹² established the pig

intestinal bacterial collection (PiBAC), a resource of cultured bacteria from the pig intestine. We evaluated the novelty of our genomes by mapping 748 genomes against these two reference datasets. The result showed that our MAGs and isolated genomes had 65.89% and 50.85% unique clusters (novel species), respectively (Fig. 2b, c and Supplementary Fig. 7a, b), which contribute new resources for the research of pig gut microbiota. In addition, most of the clusters were only detected in the MAG dataset, but not in any culture studies, reflecting the lack of culture-based studies of pig intestines.

The assembled genes contributed to the present gene catalog

We next predicted genes from the 14 metagenomic samples and generated a non-redundant gene catalog with a number of 5,283,405, which covered 22.01% of the reference gene catalog established by Xiao *et al.*⁵ (Fig. 3a, b). The samples from faeces contained the most genes, followed by the colon and ileum. Each sample contributed more than 50% of novel genes for the gene catalog. Among them, samples from ileum have the largest proportion of novel genes, followed by colon and faeces (Fig. 3a). Considering the samples of the reference gene catalog by Xiao *et al.* were derived from pig faeces, we thought that construct a more complete gene catalog of pig intestinal needs to include samples from multiple parts. Altogether, we expanded the present gene catalog to 10.91 Mb (Fig. 3b). In addition, the coverage of genes obtained by MAGs and isolated genomes were 12.97% and 1.29%, respectively, whereas 62.97% of the genes in the isolated genomes could not be detected by metagenomics (Fig. 3c). This indicates that the missed genes from metagenomic analyses can be detected by culture-dependent methods.

Functional insight of gut microbial in weanling piglets

The comprehensive gene catalog of pig intestines enables a higher-resolution functional analysis to better understand the interaction between gut microbes and pigs. We subsequently performed a KEGG pathway annotation of total protein sequences and found that 213 pathways were annotated in at least one genome. The

most general pathway was Metabolic pathways, followed by Biosynthesis of secondary metabolites, Biosynthesis of antibiotics, Microbial metabolism in diverse environments, and Biosynthesis of amino acids, which were extensively annotated in all genomes (Supplementary Fig. 8 and Supplementary Table 5). In addition, we predicted 229 carbohydrate-active enzymes (CAZymes) in all genomes to explore the ability to metabolize carbohydrates (Fig. 4a and Supplementary Table 5). The result showed that the CAZymes of bacteria are mainly glycoside hydrolases (GHs), whereas archaea are glycosyl transferases (GTs) (Fig. 4a). This indicates that bacteria obtained energy for growth by metabolizing carbohydrates, while archaea obtain energy through other pathways and synthesize sugars substance.

Antibiotics are widely used for the treatment and prevention of infection during the suckling period. Microbes may accumulate antibiotic resistance genes (ARGs) under the exposure of antibiotics, and can eventually evolve into drug resistance. We have predicted a total of 2,113 ARGs that can be classified into 38 drug classes from metagenomics, isolate genomes and MAGs (Fig. 4b). Comparison of the ARGs predicted by the three approaches showed that metagenomics can detect the largest number and types of ARGs, followed by culture-dependent, while a large number of ARGs will be lost in the process of reconstructed genomes from metagenomic bins (Fig. 4b). For analysis of virulence factors (VFs), we used the predicted genes for blast to VFDB⁴⁵. A total of 1,866 VFs were predicted in 748 genomes (Supplementary Fig. 9). Similarly, a large amount of VFs cannot be detected in MAG. We note that *Escherichia flexneri* not only had most various ARGs, but also contained a large number of VFs.

Discovery of novel SMBGs in the gut microbiome of weanling piglets

Microbes produce a series of secondary metabolites that are not necessary for life activities but have biological activity, which usually mediate important interactions between microbe and microbe-hosts. We explored secondary metabolite biosynthetic gene clusters (SMBGs) in the 748 genomes, and identified 445 SMBGs that could be classified into 19 types from 292 genomes (Supplementary Table 6). Most of these SMBGs are responsible for the synthesis of bacteriocin, followed by sactipeptide, arylpolyene, and lantipeptide (Fig. 4c). These SMBGs are predicted

from the genomes from 6 phyla, among which Firmicutes contained the largest number and widest variety of SMBGs (Fig. 4c).

For clustering the 445 SMBGs, we generated a total of 231 families, which were distributed in 5 classes of ribosomally synthesized and post-translationally modified peptides (RiPPs), terpene, non-ribosomal peptide synthetases (NRPs), polyketide synthases (PKS) and Others. RiPPs make up the largest class, but only 4 families of RiPPs that are included the reference with known functions in the MIBiG database. Salivaricin CRL1328 is a bacteriocin produced by *Ligilactobacillus salivarius*^{15, 16}, which has activity against pathogenic bacteria such as *Enterococcus faecalis* and *Enterococcus faecium*. We have predicted SMBGs related to the biosynthesis of Salivarin CRL1328 in 22 isolated *L. salivarius* genomes (Fig. 4d). We speculating that these strains have the potential to inhibit infections. Gassericin, derived from *Lactobacillus gasseri*, is another important bacteriocin. Studies have shown that Gassericin A can confer diarrhea resistance in pigs¹⁷. We discovered SMBGs that synthesize Gassericin E and Gassericin T in isolated genomes of *L. johnsonii* (Fig. 4d).

The pangenome analysis of representative species

To extend the phylogenetic analysis of representative species in the gut of weanling piglets at a genome-wide level, we conducted a pangenome analysis of 8 species with the genome number more than 10, including *Escherichia flexneri* (n=16), *Enterococcus faecalis* (n=22), *Enterococcus faecium* (n=21), which represent opportunistic pathogens, and *Lactobacillus amylovorus* (n=16), *Ligilactobacillus salivarius* (n=43), *Limosilactobacillus mucosae* (n=13), *Limosilactobacillus reuteri* (n=21), *Lactobacillus johnsonii* (n=17), which represent probiotics. As expected, the resulting accumulation curves showed that the gene repertoires of pan-genome of all the representatives were increased on addition of a new genome and the core genome decreased in contrast (Fig 5a and Supplementary Fig. 10). However, the number of gene families did not show a rapid increase in the pan genome. *E. flexneri*

275 contained the largest pan genome size of 8,143 genes, 3,111 of which formed the
276 core genome (Supplementary Fig. 11 and Supplementary Table 7). Over half of core
277 genes present in the genomes of *E. faecalis* and *E. faecium*, indicating that gene loss
278 and acquisition happened less frequently in these two species. The size of the
279 species-specific pan genomes of the 5 lactobacilli varied from 3,194 to 4,187 genes,
280 respectively (Supplementary Fig. 11 and Supplementary Table 7).

281 We next analyzed the function details of the core genomes and pan genomes,
282 including the Clusters of Orthologous Groups (COGs) and KEGG classification,
283 biosynthesis of bacteriocin, and ARGs. It is obvious that different COG functional
284 classes were enriched in both the core and pan genomes between the opportunistic
285 pathogens and probiotics. COG0438 (Glycosyltransferase involved in cell wall
286 biosynthesis), COG4690 (Dipeptidase), and COG1307 (Fatty acid-binding protein
287 DegV) were enriched in the core genomes compared to their respective accessory
288 and unique genomes of the 5 lactobacilli (Supplementary Table 8), indicating that
289 these COG categorizations represented housekeeping functions involved in
290 implementing the basic growth and metabolism of these probiotics. However, in
291 contrast to lactobacilli, *E. flexneri*, *E. faecalis*, and *E. faecium* possessed more
292 abundant COG1609 (DNA-binding transcriptional regulator, LacI/PurR family) and
293 COG1132 (ABC-type multidrug transport system, ATPase and permease component)
294 in their core genomes (Supplementary Table 8).

295 For determining the ARGs distribution in the core and pan genome of the 8 species,
296 we discovered tetracycline and macrolide were distributed in the core genome of *E.*
297 *flexneri*, *E. faecalis*, and *E. faecium*, respectively (Fig. 5b), indicating a wide
298 prevalence of tetracycline and macrolide resistance among these species. Their
299 presence may have resulted from the overuse of antibiotics in farmed pigs for
300 disease prevention in past years. Nevertheless, rare ARGs present both in the core
301 and pan genome of other lactobacilli (Fig. 5b and Supplementary Table 9), suggests
302 the safety of these species for using as probiotics in feeding of weanling piglet.

Reuterin, produced by some strains of *L. reuteri*, has antimicrobial properties¹⁸. We investigated the prevalence of biosynthesis genes (*pdu-cbi-cob-hem*) related to reuterin in the core and dispensable genomes and detected the production of reuterin experimentally. In total 41 genes related to the synthesis of reuterin were predicted in the pan genome of *L. reuteri*, including *cbiA~cbiT* (15 genes), *cobB~cobU* (10 genes), *hemA~hemL* (7 genes), and *pduA~pduV* (9 genes) (Fig. 5c and Supplementary Table 10). Notably, only *cobB*, *cobC_1*, and *cobC_2* were found in the core genomes for all the 21 genomes. The rest were present in the accessory or unique genomes. 9 of 21 genomes encoded completed biosynthesis gene clusters (*pdu-cbi-cob-hem*), indicating these strains were most likely to be reuterin producers. We next detected the production of Reuterin in the fermentation supernatant of the culture of 19 isolated *L. reuteri*. As expected, 4 strains with completed biosynthesis gene clusters (*pdu-cbi-cob-hem*) yield reuterin ranged from 0.6 to 2.54 nm/L (Supplementary Table 10).

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318 DISCUSSION

Previous culture-independent methods have uncovered that the gut microbiota is associated with the survival rate of piglet after weaning¹⁹. In this study, we investigated the gut microbiota of piglets combining with culture-based and metagenomic strategies. A collection including 1,476 cultured bacteria, 748 reference genomes catalog, comprising 266 cultured genomes and 482 MAGs, which represent the gut microbiome of piglet was constructed. 70.80% of these were represent novel species according to the present database. We also found 5 MAGs were archaea. We contributed 3,630,043 novel genes to the present pig gene catalog and expanded to 10.91 Mb. In our collection, over half of the taxa were lactobacilli, that will be a valuable resource for probiotics development and diversity study for different niche of these species.

330 We discovered 84.20% of the clusters from MAGs could not be assigned to any

known species, only 3 clusters matched 11 cultured genomes with novelty. *Prevotella*, a dominant genus in the gut of piglet, can be detected using metagenomic analysis and a total of 26 genomes from the metagenome bins were annotated as *Prevotella*, but none were included in our culture collection constructed in this study. This emphasizes the necessity of continuing effort for developing more culture methods that are appropriate for more bacteria.

Our genome-based functional analysis provides a better understanding of the gut microbiota of weanling piglets. Genes related to the metabolic pathways, biosynthesis of secondary metabolites, and Biosynthesis of antibiotics were highly enriched in all genomes. We found that the gut microbiota of weaning piglets contained a large number of glycoside hydrolase enzymes, which can be used to metabolize major dietary carbohydrates such as corn, soybean meal, wheat, and rice bran to provide most of the energy and nutrients needed for daily life²⁰. The excavation of SMBGs found antibiotics that inhibit pathogenic bacteria such as *E. faecalis* and *E. faecium*, emphasized the effect of gut microbiomes in suppressing infections in piglets. Finally, we annotated the ARGs and VFs from metagenomes and genomes. It is worth noting that the metagenomes contained abundant ARGs and VFs, which were distributed in specific isolated genomes, whereas the MAGs had a very small number of annotated ARGs and VFs. We highlighted that both techniques are complement for the detection of harmful genes.

Pan genome analyses provide comprehensive genetic landscapes of the representative species and identify the characteristics of core and strain-specific genes. *Lactobacillus*, which represent the probiotics in the pig gut, implement functions with COG0438 (Glycosyltransferase involved in cell wall biosynthesis), COG4690 (Dipeptidase), and COG1307 (Fatty acid-binding protein DegV). Bacteriocin, like reuterin, can be used as a broad-spectrum antimicrobial agent to prevent piglet diarrhea¹⁷. We discovered the completeness of biosynthesis pathway for reuterin is strain-specific, the reuterin-related genes were present in the dispensable genome, indicating when exploitation and application of bacteriocin, the

360 strain level diversity should be considered. In this collection, we identified 4/21 *L.*
 361 *reuteri* strains that had the capacity to produce reuterin, indicating those strains have
 362 potential in prevention of pathogen infections and colonization of the piglet
 363 gastrointestinal tract.

364 Metagenomic binning has been widely used to recover genomes from the fecal
 365 samples. But the cultured bacteria are of great importance for the experimental
 366 validation of their functions, especially for the development of probiotics. As a
 367 result of this study, we have provided both the cultured bacteria and reference
 368 genome data, that are publicly available at CNGB. It will be a useful resource for the
 369 future studies of microbiota–host interactions and the development as probiotics for
 370 disease prevention of weanling piglets.

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AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: Y.Z. and W.W. Performed the experiments: B.D, Y.Z., X.L, and X.J. Analyzed the data: Y.Z., X.L, T.H., J.Z., J.C, and G.L. Contributed reagents/materials/analysis tools: B.D., Y.Z., X.J., Z.C., J.L., and L.X. Supervised the work: L.X., B.W., Y.H., S.L., and J.L. Wrote the paper: B.D., Y.Z., X.L, and T.H. Revised the paper: B.D., Y.Z., and W.W. All authors commented on the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study have been deposited into CNGB Sequence Archive (CNSA)²¹ of China National GeneBank DataBase (CNGBdb)²² with accession number CNP0002075 and CNP0002072 for metagenomic and bacterial genomic data, respectively. All the bacterial strains in our collection have been deposited in China National GeneBank (CNGB), a non-profit, public-service-oriented organization in China.

ONLINE METHODS

Sample collection and bacteria culturing

42 healthy piglets (Duroc♂×(Landrace×Yorkshire)♀) with good mental state were selected for sampling, of which 12 piglets of lactation were sampled on 18 days for

543 ileum and colon content, respectively, 21 weaned piglets were sampled on 35 days for
544 ileum and colon content, and 9 weaned piglets were sampled on 60 days for faeces.
545 During sampling, the intestinal contents were obtained by dissecting the colon and
546 ileum with sterile instruments. Every 3 samples from one line with same time point
547 and region were pooled together. All samples were stored at -80 °C and prepared for
548 bacterial culturing and metagenome sequencing.

549 For bacteria culture, samples were processed according to our previous bacteria
550 culture-based study²³. To acquire more different bacterial taxa, we applied a batch of
551 rich and selective culture medium which are provided in the Supplementary method.
552 The purified bacteria were maintained with 20% glycerine at -80 °C. Detailed
553 information about the origin of all strains, including the age of the donors and gut
554 region are presented in Supplementary Data 1.

555 The acquired bacteria were subject to 16S rRNA gene sequencing. The amplification
556 of 16S rRNA gene was using 27f and 1492r primers and the obtained sequences were
557 trimmed as previously described²⁴. The taxonomy of bacteria was recognized by blast
558 the 16S rRNA gene sequences against reference sequences within the NCBI 16S
559 ribosomal RNA sequence database. We used the recommended threshold of 98.7%
560 and 94.5% as the species and genus boundaries²⁵. 16S rRNA gene sequences were
561 aligned by MAFFT v7.310²⁶ and trimmed by trimAl v1.4. rev 22²⁷ with auto option.
562 Phylogenetic tree based on 16S rRNA gene sequences was reconstructed by using the
563 maximum-likelihood method with FastTree Version 2.1.3 SSE3²⁸.

564 **Genome sequencing and assembly**

565 Genomic DNA of the 226 representative strains was extracted using the method as
566 previously described²³. The draft genomes were sequenced for 100 bp paired-end on
567 the DNBSEQ-T7 platform. The raw reads were filtered using SOAPnuke26²⁹ (v1.5.0;
568 -l 20 -q 0.4 -n 0.1 -d -M 3 --seqType 0 -Q 2 -c 2.666666666666667). After preliminary
569 assessment of the genome size based on kmer, SPAdes (v3.11.1; -T 4 -m 100) was
570 used to assembly.

571 **Metagenome sequencing, assembly, and binning**

572 Metagenomic DNA from 14 samples was extracted and sequenced according to the
573 method described above. Raw reads of each sample were preprocessed by
574 SOAPnuke (v1.5.2)²⁹ using the 'filter' module (option -l 20 -q 0.2 -n 0.05 -Q 2 -d -c
575 0 -5 0 -7 1), and host reads were removed by SOAPaligner³⁰ (v2.22, option -m 4 -s
576 30 -r 1 -n 50 -x 1000 -v 4). Kraken2³¹ was used to assign taxonomic labels to the
577 metagenome reads and calculate bacterial abundance and the profile was provided in
578 Supplementary Table 1. Shannon alpha diversity indexes ('diversity' function) and
579 Bray-Curtis dissimilarity indexes ('vegdist' function) for the principal coordinate
580 analysis (PCoA) were identified in R software with species abundances.

581 Thereafter, IDBA-UD (v 1.1.3)³² was used to assemble and merge the optimal contig.
582 MAGs were generated using three different tools configured in the 'binning' module
583 of metaWRAP (v1.1.5)³³ (integrated with MaxBin2, MetaBAT2, and CONCOCT)
584 and were finally consolidated and optimized using 'Bin_refinement'. dRep³⁴ (v2.5.4,
585 option -p 8 -comp 70 -con 5) was used to de-redundancy and preliminary quality
586 control of the MAGs. The whole-genome assembly was as described by Zou *et al*²³.
587 The quality of the MAGs and isolated genomes was estimated by CheckM
588 (v1.0.12)³⁵. The classification criteria for high-quality genomes are based on >90%
589 completeness and <5% contamination, while genomes with <70% completeness
590 or >5% contamination were not included in the following analysis.

591 **Species-level clustering, phylogenetic and taxonomic analyses**

592 FastANI (v1.32)³⁶ was used to calculate the pairwise ANI, and genomes were
593 clustered into species-level clusters with a 95% ANI cut-off using the R package
594 hclust. dRep was used to select the best genome from each cluster as the
595 representative. All genomes were taxonomically annotated using GTDB-Tk³⁷
596 (v1.3.0, database release95³⁸). Any lineage without an annotated species or invalid
597 names with reserved suffixes was considered to represent a potential new species.
598 The maximum-likelihood phylogenetic tree of the representative genomes was

constructed with GTDB-Tk ('infer' followed by 'classify_wf'). The phylogenetic tree was viewed using the online tool EVOLVIEW v2³⁹.

Alignment with other genome collections

We downloaded the genomes from two studies, which represent the reference dataset for the MAGs and the isolated genome of the pig gut microbiome. Wang *et al.*⁹ generated a total of 360 substantially complete MAGs (>70% completeness, <5% contamination) as the first metagenomic reference for swine intestinal microbiota. PiBAC¹² comprised 117 high-quality isolated genomes representing 110 species. Both datasets were downloaded from NCBI (BioProject: PRJNA494875 and PRJNA561470, respectively). We performed a pairwise ANI calculation for all genomes, and only those ANI>95% were considered to be matched.

Gene prediction and function annotation

GeneMark.hmm PROKARYOTIC (v3.38)⁴⁰ is a gene prediction program for metagenomes. Protein-coding sequences (CDS) for each genome were predicted and annotated by Prokka v1.14.6⁴¹. The metagenome non-redundancy gene catalog was generated by CD-HIT v4.6.3⁴² (cd-hit-est with option -c 0.95 defining protein identity of 95%). Based on 95% protein similarity, we clustered the gene catalog with Xiao⁵ and calculated the gene overlap of the metagenome and genome.

Functional characterization of all CDS was performed by BLAST analysis with the KEGG database (release 81)⁴³, CAZyDB⁴⁴ (downloaded from <http://www.cazy.org/>, as of April 2016), and VFDB (setB, 2021-07)⁴⁵. ARGs were identified with rgi (5.2.0) using reference data from the Comprehensive Antibiotic Resistance Database (CARD, v 3.1.2)⁴⁶.

The SMBGs were identified by using anti-SMASH (v4.2.0)⁴⁷ and were clustered by using MIBiG (version 1.4)⁴⁸ with reference BGCs by BiG-SCAPE⁴⁹ with default parameters. The similarity networks of the SMBGs were displayed by using Cytoscape (v3.8.2)⁵⁰.

626 **Pan genome analysis of the representative species**

627 The Bacterial Pan Genome Analysis tool (BPGA) pipeline⁵¹ was used for protein
 628 clustering (usearch, 80% identity value), identification of core, accessory, and
 629 unique genes of each cluster, and generation of pan-genome accumulation curves for
 630 the 171 genomes in eight clusters. Clusters of Orthologous Groups (COGs) of
 631 proteins based on Prokka annotation described above were collected to identify the
 632 core COGs specific to genome clusters with an in-house script. Total ARGs of these
 633 clusters were summarized to determine the presence and absence of them in each
 634 genome and to identify the core and dispensable genomes of antibiotic resistance
 635 with an in-house script.

636 **Detecting the production of reuterin**

637 Reuterin production from *L. reuteri* cultures was determined according to the
 638 previous described method¹⁸. In brief, cell pellets were harvested by centrifugation
 639 from overnight grown cultures of *L. reuteri* in MRS broth. The pellets were washed
 640 twice with 0.1 M potassium phosphate buffer, resuspended in 70% glycerol, and
 641 then incubated at 25°C for 2.5 h under anaerobic conditions. Quantification of
 642 reuterin in the suspension was determined by the colorimetric method as described¹⁸.

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656 **FIGURE LEGENDS**

657 **Fig. 1** | Gut microbiota composition in the ileum, colon, and faeces of weanling
658 piglets. a-b, Comparison of the phylum-level proportional abundance of microbiota.
659 c, Comparison of Shannon alpha diversity index of microbiota. d, Principal
660 coordinates analysis (PCoA) of the bacterial composition at the species level based
661 on Bray-Curtis dissimilarity.

662 **Fig. 2** | **Phylogeny of 266 genomes from isolated bacterial strains and 482**
663 **non-redundant metagenomic assembly genomes (MAGs).** a, Phylogenetic tree
664 comprising the 428 species-level clusters. Novel clusters are highlighted by red clades,
665 and the phylum is displayed in the first outer layer. Blue and orange bars in the second
666 outer layer represent the number of isolated genomes and MAGs in each cluster. **b-c**,
667 The number of MAGs and isolated genomes clusters matching PiBAC¹² and Wang *et*
668 *al*⁹, respectively.

669 **Fig. 3** | **Expansion of the present pig gene catalog.** a, Coverage of the gene catalog
670 from Xiao *et al.*⁵ by different samples from this study. The order is arranged from low
671 to high coverage, and samples from the ileum, colon, and faeces are colored as blue,
672 green, and orange, respectively. **b**, The overlap between genes in all samples (red) and
673 the Xiao *et al.* gene catalog (blue). **c**, Overlap of genes between three approaches
674 (metagenome, cultured-base, and MAGs).

675 **Fig. 4** | **Functional repertoires of 748 genomes.** a, The distribution of CAZymes
676 across different phylum. **b**, The distribution of ARGs in each sample and genome. The
677 presence and absence of ARGs is marked in blue and white, respectively. **c**, The
678 distribution of 19 different types of SMBGs at phylum level. **d**, The sequence

679 similarity network of SMBGs and the MiBiG references. Each node represents a
680 SMBG, the color of the node indicates the species from which the genome was
681 derived and the black circle indicates the reference BGC. Edges drawn between the
682 nodes correspond to pairwise distances. The figure shows only SMBGs for which
683 their distance from the reference is less than 0.3.

684 **Fig. 5 | The function distribution of genes in pangenome analysis of 8 species. a,**
685 **Accumulating fitting curves of pangenome gene family number in each cluster. b, The**
686 **distribution of ARGs present in the core genomes (pink) and dispensable genomes**
687 **(blue). b, The distribution of genes involved in the biosynthesis of reuterin in the core**
688 **genomes (pink) and dispensable genomes (blue).**

689

690 **Supplementary Figure 1 | The proportion of reads that were and were not**
691 **classified at the species level.**

692 **Supplementary Figure 2 | a-b, The comparison of genus-level proportional**
693 **abundance in the ileum, colon, and faeces of weanling piglets.**

694 **Supplementary Figure 3 | The 16S rRNA gene sequence phylogenetic tree of**
695 **1,476 strains.** Novel clusters are highlighted by red clades. Phylum, sample, and
696 region are display in the first, second, and third outer layer, respectively.

697 **Supplementary Figure 4 | The number of cultivated bacterial strains at genus**
698 **level from 14 samples.**

699 **Supplementary Figure 5 | Quality assessment of the genomes. a-b, Completeness**
700 **and contamination of 266 isolated genomes (a) and 482 MAGs (b), respectively.**

701 **Supplementary Figure 6 | The taxonomy of the 743 bacterial genomes. a-b, The**
702 **distribution of isolated genomes and MAGs across different phylum. Statistics show**
703 **the number of genomes (a) and clusters (b), respectively.**

704 **Supplementary Figure 7 | Comparison of MAGs and isolated genomes in**
705 **different quality standards. a, MAGs with >70% completeness and <5%**

706 contamination, while isolated genomes with >90% completeness and <5%
707 contamination. **b**, Both MAGs and isolated genomes are high quality (with >90%
708 completeness and <5% contamination).

709 **Supplementary Figure 8 | The distribution of KEGG pathways in each genome.**
710 The presence and absence of each pathway is indicated by blue and white,
711 respectively.

712 **Supplementary Figure 9 | The distribution of VFDB in each genome.** The
713 presence and absence of VFDB is indicated by blue and white, respectively.

714 **Supplementary Figure 10 | The accumulation curves of the pan and core genome**
715 **of the 8 representative clusters.** Box plots indicate 25th and 75th percentiles with
716 medians shown as horizontal lines and whiskers set at 10th and 90th percentiles.

717 **Supplementary Figure 11 | The gene family numbers of pan genome, core**
718 **genome and average genome of the 8 representative clusters.**

Figure 1

a**Phylum**

Firmicutes
Bacteroidota
Proteobacteria
Spirochaetota
Actinobacteriota
Campylobacterota
Verrucomicrobiota
Desulfobacterota
Fusobacteriota
Myxococcota

Number of genomes

Isolated genomes
MAGs

b **c** **Figure 2**

a **b** **c** **Figure 3**

a

CAZymes		AAs	CEs	GTs	CBMs	GHs	PLs
Type		Meta		Isolated genomes		MAGs	
Phylum		Actinobacteriota		Bacteroidota		Campylobacterota	
		Desulfobacterota		Firmicutes		Fusobacteriota	
		Methanobacteriota		Myxococcota		Proteobacteria	
		Spirochaetota		Verrucomicrobiota		Thermoplasmata	

C

b

d

Figure 4

Figure 5