

**High-Throughput Culturing and Genomic Characterization of Swine Gut
Bacteria: Insights into Antimicrobial Biosynthesis and Therapeutic Potentials**

By

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**A Thesis Submitted to The Faculty of Graduate and Postdoctoral Studies of
The University of Manitoba**

**In Partial Fulfillment of the Requirements of the Degree of
MASTER OF SCIENCE**

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Winnipeg, Manitoba, Canada**

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Abstracts

The swine gut microbiota plays a central role in host nutrition, immune development, and resistance to enteric diseases. However, a large proportion of swine-associated microorganisms remain poorly characterized due to the limited availability of cultured representatives and high-quality reference genomes. This knowledge gap restricts our ability to define microbial functional capacity and to identify microbial traits associated with host health and disease. In particular, post-weaning diarrhea remains a major challenge in swine production, contributing to impaired animal performance, economic losses, and increased antimicrobial use. Therefore, improving our understanding of the functional ecology of the swine gut microbiome is essential for the development of sustainable microbiome-based strategies to enhance animal health and disease resilience.

In this thesis, high-throughput culturomics and whole-genome sequencing approaches were used to establish a comprehensive collection of bacterial isolate genomes from the swine gastrointestinal tract and feces. The resulting genome resource consisted of 300 high-quality, non-redundant bacterial genomes spanning diverse phylogenetic groups, including several previously uncultured or underrepresented taxa. Comparative genomic analyses identified 55 putatively novel species and revealed extensive functional diversity within carbohydrate-active enzymes (CAZymes), metabolic pathways, biosynthetic gene clusters (BGCs), and antimicrobial peptides (AMPs). Functional features associated with secondary metabolite production and antimicrobial activity were particularly enriched among several health-associated bacterial lineages. To evaluate the ecological relevance of these functional traits, metagenomic datasets from independent pig cohorts with and without post-weaning diarrhea were analyzed using genome-resolved read recruitment approaches. Functional signatures related to BGCs and AMPs consistently differentiated healthy pigs from diarrhea-associated animals, with reduced abundance and diversity observed in diseased microbiomes. Several bacterial genera, including *Blautia*, *Dorea*, *Prevotella*, *Parabacteroides*, *Paratractidigestivibacter*, and *Aneurinibacillus* emerged as important contributors to microbial functions associated with healthy gut microbiota and depleted in post-weaning diarrhea.

This work expands the current genomic representation of the swine gut microbiome and provides new insights into the functional potential of cultured swine-associated bacteria. The findings highlight the importance of microbial biosynthetic and antimicrobial capacities in maintaining gut health and identify potential microbial biomarkers and therapeutic candidates for reducing susceptibility to gastrointestinal diseases in swine production systems.

Keywords: Swine gut microbiota, Culturomics, Biosynthetic gene clusters, Antimicrobial peptides, Post-weaning diarrhea, Functional genomics, Microbiome-host interactions

Acknowledgments

I would like to express my sincere gratitude to my advisor, Dr. Hooman Derakhshani, for his continuous support, guidance, and mentorship throughout my M.Sc. program. Your encouragement, patience, and invaluable insights have played a significant role in both my academic and personal development. It has been a true privilege to work under your supervision, and I deeply appreciate the trust and confidence you placed in me throughout this journey. I am truly grateful for your unwavering support and inspiration, without which this achievement would not have been possible.

I would also like to extend my appreciation to my advisory committee members, Dr. Sean Walkowiak and Dr. Chengbo Yang, for their valuable feedback, thoughtful suggestions, and guidance during committee meetings, which greatly contributed to the progress of my research. In addition, I would like to thank the swine barn staff at Glenlea Research Station, for their assistance and support in conducting this trial.

Special thanks go to my lab mates and friends, Dr. Zisanur Rahman, Arezoo Alizadeh, Nahidur Rahman and Larissa Scott, for creating such a supportive and enjoyable environment. Your friendship and encouragement made this journey far more meaningful.

I would also like to express my heartfelt gratitude to my husband and my parents for their unconditional love, endless encouragement, and constant support throughout every stage of my life. I am truly fortunate to have you by my side.

Finally, I gratefully acknowledge the financial support provided by the NSERC CRSNG, RBC scholars, Sustainable Canadian Agricultural Partnership and the University of Manitoba, which made this research possible.

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List of Abbreviations

FAO	Food and Agriculture Organization of the United Nations
OECD	Organisation for Economic Co-operation and Development
PRRS	Porcine Reproductive and Respiratory Syndrome
ASF	African Swine Fever
FMD	Foot and Mouth Disease
PEDV	Porcine Epidemic Diarrhea Virus
PDCoV	Porcine Deltacoronavirus
PCU	Population Correction Unit
AMR	Antimicrobial Resistance
IL	Interleukin
TNF- α .	Tumor Necrosis Factor alpha
HPA	Hypothalamic–Pituitary–Adrenal
GLP-1	Glucagon-Like Peptide-1
PYY	Peptide YY
CCK	Cholecystokinin
SCFAs	Short-Chain Fatty Acids
FXR	Farnesoid X Receptor
GALT	Gut-Associated Lymphoid Tissues
PRRs	Pattern Recognition Receptors
TLRs	Toll-like Receptors
NLRs	NOD-like receptors
IgA	Immunoglobulin A
MNPs	Microbial Natural Products
BGCs	Biosynthetic Gene Clusters
RiPPs	Ribosomally Synthesized Peptides
PKs	Polyketides
NRPs	Nonribosomal Peptides
CAZymes	Carbohydrate-Active Enzymes
MAG	Magenome-Assembled Genome

PIGC	Pig Integrated Gene Catalog
UPGG	Unified Pig Gastrointestinal Genome
TH17	T Helper 17
ETEC	Enterotoxigenic <i>Escherichia coli</i>
AGPs	Antimicrobial Growth Promoters
FOS	Fructooligosaccharides
GOS	Galactooligosaccharides
ZnO	Zinc oxide
Tregs	Regulatory T cells
NK	Natural Killers
MAMPs	Microbe-Associated Molecular Patterns
ECs	Epithelial Cells
AMPs	Antimicrobial Peptides
ILCs	Innate Lymphoid Cells
AhR	Aryl hydrocarbon Receptor
DCs	Dendritic Cells
GPCRs	G-Protein–Coupled Receptors
HDACs	Histone Deacetylases
SFB	Segmented Filamentous Bacteria
Muc2	Glycoprotein Mucin 2
LCN2	Lipocalin 2
fhuA Receptor	Ferrichrome Outer Membrane Transporter
MRSA	Methicillin-Resistant <i>Staphylococcus aureus</i>
VRE	Vancomycin-Resistant <i>Enterococcus</i>
PRSP	Penicillin-Resistant <i>Streptococcus Pneumonia</i>
CA	Cholic Acid
CDCA	Chenodeoxycholic Acid
DCA	Deoxycholic Acid
LCA	lithocholic Acid
PKSs	Polyketide Synthases
HGT	Horizontal Gene Transfer

HMM	Hidden Markov Model
antiSMASH	Antibiotics & Secondary Metabolite Analysis Shell
BiG-SCAPE	Biosynthetic Gene Similarity Clustering and Prospecting Engine
GCFs	Gene Cluster Families GCFs
PMGCs	Primary Metabolic Gene Clusters
FMT	Fecal Microbial Transplantation

1. Chapter 1: General Introduction

Pigs are among the most crucial livestock species, serving as a primary source of meat for human consumption alongside poultry (1). The pork industry is a critical component of Canada's agricultural sector, ranking as the fourth-largest farming industry in the country. It plays a significant role in strengthening the national economy, generating jobs in both rural and urban regions, and supplying Canadians, as well as millions globally, with affordable, high-quality protein (2). In addition, global demand for meat is expected to rise substantially over the next two decades, driven by population growth. According to projections from the Food and Agriculture Organization of the United Nations (FAO) (3) and the Organisation for Economic Co-operation and Development (OECD) (4), global meat consumption is forecast to increase by nearly 48 million tonnes by 2034, with per capita consumption rising as diets diversify and economic development expands. This trend reflects broader patterns of increasing demand for animal-source foods, including beef, pork, and poultry, which together support nutritional needs in emerging markets and contribute to sustained growth in global livestock production systems. Pig meat in particular is projected to remain a key component of this rising demand, with global pork supply expected to grow steadily into the 2030s as production recovers from disease outbreaks such as African Swine Fever (ASF) and expands in response to higher consumer appetite in developing regions (4).

Sustainable pig production faces major challenges in the context of increasingly intensive farming systems, persistent infectious diseases, and long-standing reliance on antimicrobials. The globalization and intensification of pig production have greatly increased animal density and movement of pigs, feed, and pork products, creating ideal conditions for the rapid emergence and spread of pathogens such as porcine reproductive and respiratory syndrome (PRRS), African swine fever (ASF), foot and mouth disease (FMD), and influenza (5,6). These diseases have caused severe economic losses and continue to threaten food security. In addition, gastrointestinal diseases have long posed a significant challenge for Canadian farmers, particularly in the pork industry. For example, viral infections such as porcine epidemic diarrhea virus (PEDV) and porcine deltacoronavirus (PDCoV), are known to cause severe illness and death in swine, leading to substantial economic losses (7). Beyond viral pathogens, bacterial

enteric diseases remain among the most important causes of morbidity and production losses in swine herds worldwide. In particular, post-weaning diarrhea (PWD), primarily associated with enterotoxigenic *Escherichia coli* (ETEC), is one of the most common and economically significant diseases affecting nursery pigs, leading to reduced growth performance, increased mortality, and frequent antimicrobial interventions (8). Similarly, swine dysentery, caused by *Brachyspira hyodysenteriae*, results in severe mucohemorrhagic diarrhea, poor feed conversion, and chronic herd-level productivity losses, particularly in growing-finishing pigs (9). Other important bacterial enteric pathogens, including *Lawsonia intracellularis* (ileitis) and *Salmonella enterica*, further contribute to the complex multifactorial nature of porcine gastrointestinal disease syndromes (10).

Antimicrobial agents have traditionally been widely used in swine production to manage infectious diseases, promote growth, and prevent illness. However, the extensive and often indiscriminate use of antibiotics has contributed to the rise of antimicrobial resistance (AMR). In Canada, swine production represents the largest user of medically important antimicrobials in animal agriculture, accounting for approximately 257 mg/PCU and ranking as the number one buyer among livestock sectors (11). This growing resistance poses serious threats to both human and animal health, as it reduces the effectiveness of treatments and increases the risk of resistant infections spreading, ultimately leading to deteriorating health outcomes in both populations (12–14).

Given these constraints, there is an urgent need to develop alternative strategies that support pig health and productivity while reducing dependence on antimicrobials. Sustainable solutions must enhance disease resistance, improve feed efficiency, and maintain animal performance under intensive and climate-challenged conditions without exacerbating AMR risks. Approaches such as improved biosecurity, vaccination, nutritional interventions and microbiome-based strategies are increasingly viewed as essential tools for modern pig production systems (15,16). In particular, manipulating the gut environment to promote beneficial microbial populations and nutritional interventions and feed additives such as probiotics, prebiotics, organic acids, plant extracts represent a promising approach to improve immune function, growth performance, and resilience to pathogens (15,17). Therefore, identifying safe, effective, and economically viable alternatives to antibiotics is not only critical

for maintaining competitiveness in the pork industry but also for protecting public health and ensuring the long-term sustainability of global pig production.

The gut microbiota consists of diverse living microorganisms, including bacteria, fungi, viruses, archaea, and protozoa, that inhabit specific niches along the GI tract. They cover nearly all mucosal surfaces of their host, such as the oral cavity, lungs, skin, but are found at a higher density in the gastrointestinal system that plays a crucial role in regulation of both the immune system and metabolic homeostasis (18,19).

The gut microbiota is dominated by anaerobic bacteria, which are far more prevalent than aerobic lineages. Swine gut microbiota is dominated by the phyla Bacillota (formerly Firmicutes), Bacteroidota (formerly Bacteroidetes), and Pseudomonadata (formerly Proteobacteria). At the genus level, taxa such as *Clostridium*, *Lactobacillus*, *Prevotella*, *Ruminococcus*, and *Blautia* are consistently present in healthy pigs and contribute to a stable, autochthonous microbiota that supports host health and resistance to pathogen colonization (20). The term microbiome has a broader definition and refers to the genetic material of all living microorganisms within a community. The microbiome contains genes coding a variety of structural elements such as proteins, lipids, polysaccharides, , metabolites, signaling molecules and toxins (19,20). Due to its massive genetic repertoire, the gut microbiota performs various functions essential for maintaining health, immune and metabolic homeostasis. These include aiding digestion by producing key enzymes that can break down indigestible polysaccharides. Additionally, the microbiota contribute to the training and regulation of immune responses, strengthens the intestinal epithelial barrier, and helps prevent the colonization of pathogens (21,22). In addition, the gut microbiota is connected to the central nervous system through a complex bidirectional communication network known as microbiota–gut–brain axis, regulating gut physiology, immunity, metabolism, and behavior (23). This interaction is mediated through multiple interconnected pathways, including neural routes such as the autonomic and enteric nervous systems, spinal afferent nerves, and the vagus nerve, which transmit sensory and regulatory signals between the gut and brain (23,24). Immune signaling also plays a central role, as microbial–host interactions shape cytokine production including interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor alpha (TNF- α) and inflammatory tone, with downstream effects on brain function (23,25).

The gut microbiota enhances feed efficiency in pigs through its extensive repertoire of carbohydrate-active enzymes (CAZymes), which degrade otherwise indigestible dietary polysaccharides (e.g., cellulose, hemicellulose, and resistant starch) into fermentable substrates (26–28). These substrates are further metabolized by microbes into short-chain fatty acids (SCFAs), primarily acetate, propionate, and butyrate, which serve as key energy sources and regulate host lipid and glucose metabolism. For example, *Ruminococcus* spp. and *Prevotella* spp. contribute to fiber degradation and SCFA production (29). In addition, microbial biosynthetic pathways contribute to the production of metabolites such as vitamins, and bile acid derivatives that enhance energy harvest and regulate metabolic pathways, contributing to improved growth performance and feed conversion (30).

The gut microbiota is also essential for the maturation and proper functioning of both the intestinal immune system and the physical gut barrier. Early microbial colonization drives the development of intestinal structures such as villus vasculature, mucus production, and gut-associated lymphoid tissues (GALT), while also shaping immune cell populations in the lamina propria and mesenteric lymph nodes. By continuously interacting with epithelial cells and immune receptors like pattern recognition receptors (PRRs) and toll-like receptors (TLRs), commensal microbes stimulate cytokine production, regulate innate immune signaling, promote mucin secretion, and induce antimicrobial peptides that help maintain spatial separation between microbes and the epithelium. The microbiota also supports adaptive immunity by guiding dendritic cell-mediated T cell differentiation and promoting immunoglobulin A (IgA) production, which neutralizes pathogens in the intestinal lumen (31). In addition to strengthening host immunity, the gut microbiota also provides colonization resistance against environmental pathogen and opportunistic microbes through both direct and indirect mechanisms competing with pathogens over nutrient and physical niches, producing bacteriocins and inhibitory metabolites such as secondary bile acids and SCFAs, reinforcing the mucus barrier, and maintaining an oxygen-limited environment that suppresses the growth of facultative anaerobic pathogens (32).

The gut microbiota harbors hundreds of bacterial species that are capable of producing various bioactive compounds, referred to as secondary metabolites, also known as microbial

natural products (MNPs), which play a role in mediating microbe-host and microbe-microbe interactions within the gut ecosystem (33,34). These natural products are encoded by specific genes grouped together in clusters, known as biosynthetic gene clusters (BGCs), which can be found in either the bacterial chromosome or plasmids (34,35). There is a wide variety of MNPs with a wide range of targets that can enhance or modify the host's immune responses and other physiological processes. These molecules can be categorized into several classes; some of the most important classes of secondary metabolites include Ribosomally synthesized and post-translationally modified peptides (RiPPs) including bacteriocins, lantibiotics and microcins (36), amino acid metabolites including indole, tryptamine (36,37), oligosaccharides such as polysaccharide A (38), glycolipids (36,39), terpenoids including secondary bile acids (36,40,41), polyketides (PKs) and nonribosomal peptides (NRPs) including cereulide, mycolactone, colibactin, and tilivalline (36).

Over the past decade, culture-independent sequencing approaches, including 16S rRNA gene amplicon sequencing and shotgun metagenomics, have revolutionized our understanding of the taxonomic composition and functional potential of gut microbial communities. These approaches have revealed core microbial taxa and metabolic pathways associated with host health, performance, and disease susceptibility (42,43). However, sequencing-based methods are largely correlative and rely heavily on reference databases, limiting their ability to assign functions to specific organisms or the possibility to experimentally validate predicted activities. As a result, there remains a substantial gap between genomic predictions and mechanistic understanding of microbial function (44).

Culturomics is a powerful complementary approach that addresses these limitations by enabling large-scale isolation of gut microbes under diverse culture conditions. By systematically varying growth media, incubation times, temperatures, and atmospheric conditions, culturomics dramatically expands the diversity of microorganisms that can be recovered from complex ecosystems (45). Importantly, the availability of living isolates provides direct access to microbial physiology, metabolism, and bioactive compound production, allowing functional validation of genomic predictions (42,44).

Recent advances in high-throughput culturomics combined with bioinformatic platforms for functional annotation of microbial genes have facilitated the systematic exploration of the mammalian gut microbiota for previously uncharacterized bioactive metabolites. These approaches have enabled the discovery of BGCs involved in the production of secondary metabolites, including NRPs, PKs, bacteriocins, and other bioactive compounds, many of which exhibit antimicrobial, immunomodulatory, or signaling properties that influence microbial competition and host responses (46,47). Notably, genome-guided mining of the human microbiome has led to the identification of novel antibiotics such as humimycins, which potentiate the activity of existing antimicrobials against methicillin-resistant *Staphylococcus aureus* in preclinical mouse models (46). Similarly, thiopeptides such as lactocillin, produced by *Lactobacillus gasseri*, demonstrate selective antimicrobial activity while preserving commensal microbial communities, highlighting the translational potential of microbiome-derived compounds (48). Recent studies have also revealed numerous antimicrobial peptides and natural products from both human and murine gut microbiomes, providing a rich source of narrow-spectrum therapeutics for pathogen control (49).

Despite substantial progress in cataloging the genetic and functional diversity of the pig gut microbiome, major limitations persist in the availability of comprehensive biobanked isolates and high-quality reference genomes from swine intestinal microbes. Although large metagenome-assembled genome (MAG) resources such as the pig integrated gene catalog (PIGC) and the unified pig gastrointestinal genome (UPGG) have expanded genomic resources, they also highlight that a significant portion of the microbiota remains poorly characterized, with many species lacking cultured representatives and high-quality genomes suitable for functional validation (50). Existing collections of cultured swine gut bacteria are still limited in species and strain diversity, and the functional repertoires of many isolates have not been thoroughly explored, constraining efforts to link genomic potential to phenotypes relevant for health and production (51). This gap restricts the discovery and development of probiotics, postbiotics, and other microbiome-derived therapeutics that could improve intestinal health, enhance resistance to pathogens, and support sustainable production in swine systems (52). Establishing extensive culture collections, biobanks, and high-quality genomes from diverse swine microbiota will therefore be essential to enable genome-informed discovery and

translational applications that advance both basic microbiome science and practical interventions in pig health and productivity.

2. Chapter 2: Literature Review

2.1 Introduction

The gut microbiota plays a central role in host physiology, modulating a broad range of biological functions through direct stimulation, competitive inhibition, or metabolite-mediated signaling (53). It supports intestinal health by aiding in the maturation of GALT (53), promoting tissue regeneration and gut motility (54), and strengthening the intestinal barrier by reducing epithelial permeability (55). Additionally, the microbiota contributes to the development of the vascular system around the gut, initiating processes that lead to increased blood vessel formation (56,57). Beyond these localized roles, the gut microbiota exerts systemic effects on host metabolism by fermenting dietary substrates into bioactive metabolites such as short-chain fatty acids, which influence energy harvest, lipid metabolism, and glucose homeostasis (56,58). Microbial products also interact with host endocrine pathways, modulating the secretion of hormones and gut peptides that regulate appetite, insulin sensitivity, and glucose metabolism, thereby integrating microbial signals with endocrine control of metabolism (59). In addition, the existence of a gut-lung axis, in which metabolites and immune signals derived from the intestinal microbiota reach the lungs via the circulation and lymphatic system to shape pulmonary immunity and inflammatory responses (60). Gut-derived microbial components and metabolic products, particularly SCFAs modulate both innate and adaptive immune cells in the airways, influencing tolerance to inhaled allergens and resistance to respiratory infections. For example, SCFAs increase the intestinal epithelial barrier function through increasing mucus production by goblet cells (58,61). Dysbiosis of the intestinal microbiota, such as that induced by antibiotics or reduced microbial diversity early in life, disrupts this crosstalk and increases susceptibility to allergic airway diseases and viral and bacterial pulmonary infections. Moreover, chronic gastrointestinal disorders are associated with higher prevalence of lung diseases, further highlighting the bidirectional impact of gut–lung communication on respiratory health and disease outcomes (58,62).

2.2 Defining Gut Health

Gut health is a multifaceted concept that reflects the optimal functioning and stability of the entire gastrointestinal tract. According to the study by Bischoff (63), gut health encompasses efficient digestion and nutrient absorption, the absence of gastrointestinal symptoms or diagnosed disease, a resilient epithelial barrier, and a balanced intestinal microbiota that supports immune and metabolic homeostasis. Although the term is widely used in medicine and the food industry, its definition remains imprecise due to the complexity of the digestive system and the difficulty of measuring many gut functions directly (63). A fundamental concept in gut health is the dynamic interaction between the intestinal barrier and the resident microbiota, as disruption of this relationship, commonly referred to as dysbiosis, can increase susceptibility to infectious, inflammatory, functional, and systemic disorders (64,65). Therefore, maintaining gut health involves preserving barrier integrity, supporting a stable microbiome, and preventing early signs of dysfunction, making it an essential component of preventive medicine (65).

2.3 Increasing Prevalence of Gastrointestinal Diseases and Their Impact on Gut Health in Swine

Swine infectious diseases represent a major and persistent challenge to global pork production, with significant implications for animal health, farm profitability, and food security. As emphasized in the editorial by Mo Zhou and colleagues, (66) the epidemiological landscape of swine diseases has become increasingly complex due to intensified production systems, globalization of trade, and the continuous evolution of pathogens. Modern swine farming is characterized by high stocking densities, early weaning, large herd sizes, and frequent animal movement, all of which facilitate the rapid transmission and persistence of infectious agents within and between farms (5,6,67).

Gastrointestinal diseases are among the most economically significant conditions affecting the swine industry. Post-weaning diarrhea, primarily associated with enterotoxigenic *Escherichia coli* (ETEC), remains one of the most damaging enteric disorders during the critical weaning transition (68). In addition to ETEC, several other bacterial pathogens are prevalent in commercial herds, including *Clostridium perfringens*, a cause of neonatal enteritis (69); *Lawsonia intracellularis*, responsible for proliferative enteropathy (70); *Brachyspira hyodysenteriae*, the etiological agent of swine dysentery (71); and *Salmonella enterica*, which

presents both production and food safety risks (72). Viral enteric pathogens such as porcine epidemic diarrhea virus (PEDV) and porcine deltacoronavirus (PDCoV) can also cause severe diarrhea, dehydration, and high mortality in neonatal piglets, leading to substantial economic losses (73). The epidemiology of these infections is intrinsically linked to intensive farming practices. Continuous-flow production systems and dense animal populations create ideal conditions for pathogen amplification and environmental persistence, increasing the likelihood of recurrent outbreaks (6). Gastrointestinal infections in pigs not only cause acute clinical disease but also profoundly disrupt gut microbiota composition and intestinal homeostasis, with lasting consequences for animal health and performance (74). In particular, infection with ETEC has been shown to reduce microbial diversity and promote dysbiosis, characterized by an overrepresentation of Proteobacteria and a decline in beneficial commensals such as *Lactobacillus* and *Prevotella* spp (74,75). This microbial imbalance is closely associated with increased intestinal inflammation, as ETEC virulence factors stimulate the production of pro-inflammatory cytokines such as TNF- α and IL-6 and disrupt epithelial barrier integrity (76). Consequently, nutrient absorption is impaired, and energy is diverted toward immune responses rather than growth, leading to reduced average daily gain and poorer feed efficiency in affected piglets (77).

2.4 Widespread Use of Antimicrobials for Controlling GIT Infections in Swine

Antibiotics are widely used in the swine industry for three primary purposes: growth promotion, prophylaxis/metaphylaxis, and therapeutic treatment. The historic use of antimicrobial growth promoters (AGPs) in livestock raised significant concerns regarding AMR crisis as it involved continuous inclusion of low, subtherapeutic levels of antibiotics (often belonging to classes critical for human medicine) in pig feed, creating ideal conditions for selection and dissemination of antimicrobial-resistant bacteria in the gut. Although AGPs were banned in the European Union in 2006 and restricted in many other countries, preventive metaphylactic and prophylactic use still commonly relies on in-feed antibiotics, typically administered at higher, often therapeutic, concentrations. These regimens are intended for short-term disease prevention but can be repeatedly or excessively applied. A broad range of antibiotics are also used therapeutically (78). Patterns of antimicrobial use vary internationally (78), with tetracyclines, penicillins, ceftiofur, chlortetracycline, and tylosin being among the

most frequently reported classes in North America. This extensive and often indiscriminate use of antibiotics has contributed to the rise of AMR which has become a major concern due to its implications for both animal and human health. While antibiotic use in human medicine remains the primary driver of clinically significant AMR, zoonotic pathogens, including *Salmonella*, *Campylobacter*, *E. coli*, and *Enterococcus*, are of particular concern because their exposure to veterinary antimicrobials spans drug classes also critical to human therapeutics (79,80). Among swine pathogens, ETEC strains, the leading cause of gastrointestinal disease, are of added concern given the breadth of antimicrobial resistance documented in this pathotype. *E. coli* isolates frequently display resistance across multiple drug classes, including aminoglycosides (spectinomycin, apramycin, neomycin) and potentiated sulfonamides (trimethoprim-sulfamethoxazole) (1). Of particular concern is the emergence of colistin resistance. In 2015, Liu et al. (79) identified the plasmid-mediated colistin resistance gene *mcr-1* in *E. coli* isolates from pigs, retail meat, and humans in China, strongly implicating swine production as a reservoir and demonstrating the horizontal transmissibility of resistance to a last-resort antimicrobial. The discovery of *mcr-1* marked a pivotal moment in the global AMR crisis, as it revealed that resistance to colistin could disseminate via mobile genetic elements rather than solely through chromosomal mutation (80).

This growing resistance poses serious threats to both human and animal health, as it reduces the effectiveness of treatments and increases the risk of resistant infections spreading, ultimately leading to deteriorating health outcomes in both populations (12,81). As a result, it is crucial to explore alternative strategies that can enhance disease resistance in swine. Developing such approaches is essential to curbing antimicrobial resistance and promoting more sustainable livestock practices that safeguard both animal and human health.

In response to increasing concerns over antimicrobial resistance, alternative non-antibiotic strategies have been widely explored to control gastrointestinal diseases in swine, among which pharmacological zinc oxide (ZnO) has been one of the most extensively implemented approaches. Pharmacological levels of ZnO have also been extensively used in pig production to control enteric disorders and improve intestinal health. Zinc enhances villus morphology and enterocyte proliferation, increases tight junction protein expression such as occludin, reduces intestinal permeability, modulates cytokine responses, and exhibits antibacterial activity,

particularly against Gram-positive pathogens (82–85). Moreover, ZnO improves nutrient digestibility and exerts antioxidant effects (83). Despite these benefits, prolonged high-dose ZnO use has raised significant concerns. Toxicity risks to liver and kidney tissues have been reported at excessive levels (83,86), and environmental accumulation through manure application can increase zinc concentrations in soil and groundwater to harmful levels (83,87). Importantly, therapeutic ZnO doses have been associated with increased abundance of tetracycline- and sulphonamide-resistance genes in Gram-negative bacteria and with the co-selection of heavy metal resistance determinants (88–90). Co-selection refers to the simultaneous selection of multiple resistance traits when genes conferring resistance to different stressors (e.g., antibiotics and heavy metals) are physically linked on the same mobile genetic element, such as plasmids, transposons, or integrons. In such cases, exposure to one selective agent (e.g., zinc) can indirectly enrich for bacteria carrying linked antibiotic resistance genes, even in the absence of antibiotic pressure (91). In contrast, cross-resistance occurs when a single genetic or physiological mechanism confers reduced susceptibility to multiple antimicrobial agents, for example through multidrug efflux pumps or shared detoxification pathways that reduce intracellular accumulation of both metals and antibiotics (91). Furthermore, ZnO has also been shown to alter gut microbiota composition, reducing *Lactobacillus* populations while increasing Clostridiales and Enterobacteriales diversity (83,92).

2.5 Alternative Therapeutics to Enhance Gut Health and Improve Resilience to Gastrointestinal Infections

The growing limitations of conventional antimicrobial use have prompted the exploration of alternative strategies to control enteric infections and improve gut resilience in swine production systems. Among these, microbiome-targeted nutritional interventions have gained particular attention due to their ability to modulate intestinal ecology and host physiology. For example, dietary organic acids are widely used to acidify the gastrointestinal tract, thereby inhibiting the proliferation of pathogenic bacteria and improving nutrient digestibility and growth performance (93,94). In addition, phytogenic feed additives, including essential oils and plant-derived bioactive compounds (e.g., thymol, carvacrol, and eugenol), have demonstrated antimicrobial, anti-inflammatory, and antioxidant properties, contributing to improved gut

health and performance (95,96). moreover, prebiotics stimulate beneficial microbial populations or their metabolites. For instance, prebiotics, such as inulin, fructooligosaccharides (FOS), and galactooligosaccharides (GOS), selectively stimulate the growth and metabolic activity of beneficial gut microorganisms, leading to increased production of short-chain fatty acids that support epithelial integrity and immune function (97,98). In addition, probiotics, including strains of *Lactobacillus*, *Bifidobacterium*, and *Bacillus*, contribute to gut health through competitive exclusion of pathogens, enhancement of mucosal barrier function, and modulation of both innate and adaptive immune responses (99,100).

More recently, postbiotics which are defined as non-viable microbial cells, metabolites, or cell components, have emerged as promising alternatives due to their stability and safety profile. These compounds can exert immunomodulatory, anti-inflammatory, and barrier-protective effects without the risks associated with administering live microorganisms (101,102). Collectively, these microbiome-modulating strategies contribute to improved gut microbiota stability, enhanced resilience against enteric pathogens, and maintenance of immune homeostasis (103).

2.6 Crosstalk Between the Microbiota and Immune System

The gut microbiota constitutes a highly complex and dynamic microbial ecosystem that is essential for host physiology, substantially influencing both local and systemic immune processes. Microbial communities residing in the gastrointestinal tract engage in continuous bidirectional communication with the host immune system, shaping immune development, calibrating immune responses, and maintaining immune homeostasis throughout life (104). This microbiota–immune system crosstalk encompasses both innate and adaptive immunity, where microbial signals actively regulate immune cell differentiation, cytokine production, and the balance between tolerance and inflammation (105). Gut microbiota–derived metabolites such as SCFAs modulate the function of macrophages and dendritic cells and secondary bile acids serve as key modulators of signaling pathways in immune cells, contributing to the establishment of mucosal tolerance and systemic immune regulation (104,106).

Beyond metabolite-mediated signaling, the gut microbiota directly instructs immune architecture by guiding the maturation and functional specialization of lymphoid and myeloid cell populations. Commensal microorganisms contribute to the development of GALT and promote the differentiation of naïve T cells into functionally distinct subsets, including regulatory T cells (Tregs) and effector T helper lineages, thereby maintaining the equilibrium between immune tolerance and protective immunity (104,107). At the mucosal surface, microbial cues including microbe-associated molecular patterns (MAMPs) and microbiota-derived metabolites stimulate epithelial and immune cells to produce secretory IgA, antimicrobial peptides, and mucus, reinforcing the intestinal barrier and limiting microbial translocation (108). Importantly, commensals also prevent pathogen overgrowth through competitive exclusion and production of antimicrobial factors, thereby indirectly shaping immune tone (104). Evidence from germ-free animal models shows that, in the absence of commensal microbes, key features of intestinal structure (including villus capillaries and lymphoid follicles) remain underdeveloped, and immune cell populations in the lamina propria and mesenteric lymph nodes are markedly reduced (31,109).

2.6.1 Role of Microbiota in Development of Innate Immunity

The gut microbiota plays a central role in shaping and regulating the innate immune system, promoting a balanced environment that supports both microbial survival and host defense. This relationship is mediated by microbial components (e.g., MAMPs), metabolites (e.g., SCFAs), and epithelial signals that together regulate cellular differentiation, barrier integrity, cytokine networks, and innate effector functions (104,110). For example, the gut microbiota controls mucus production through regulation of goblet cell differentiation and mucin gene expression (e.g., MUC2). Certain commensals, including *Akkermansia muciniphila* and members of the *Lachnospiraceae*, induce goblet cell expansion and mucin secretion, reinforcing the physical barrier that separates luminal microbes from the epithelium. The mucus layer also acts as a reservoir for antimicrobial peptides and microbial metabolites, facilitating controlled host–microbe interactions (111). The gut microbiota also influences immune cell differentiation and helps maintain a balanced interaction between the host and the microbiota. For instance, after birth, specific immune cells, including ROR γ t⁺NKp46[–] LTi-like cells, accumulate in the gut and can differentiate into natural killers (NK)-like cells, which are distinct from regular NK

cells (112). This process requires the presence of the intestinal microbiota and the cytokine IL-23. These NK-like cells produce IL-22, which supports intestinal barrier integrity and reduces bacterial infiltration by promoting epithelial repair (53). Additionally, gut microbiota impacts the production of immune mediators, such as cytokines, chemokines, and other soluble factors. The gut microbiota is essential in modulating the immune response by influencing the T cell population and other immune elements (55). In particular, the microbiota encourages B cells to differentiate into IgA-producing plasma cells (18,113), which are critical for preserving the intestinal barrier and preventing bacterial translocation (114,115).

2.6.1.1 Gut-Associated Lymphoid Tissue (GALT) and Microbiota-Driven Innate Programming

GALTs, including Peyer's patches, isolated lymphoid follicles, and mesenteric lymph nodes, serve as immunological hubs where commensal signals integrate with innate immune cell development. Colonization by commensal bacteria is essential for the structural and functional maturation of these tissues: germ-free animals show markedly reduced lymphoid follicle development and impaired immune surveillance (104). Mechanistically, pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) and NOD-like receptors (NLRs) expressed on epithelial cells (ECs) and innate immune cells recognize MAMPs (113). These receptor–ligand interactions activate downstream signaling pathways, including NF- κ B and MAPK, which drive production of AMPs, chemokines, and cytokines that condition local innate responses and recruit additional immune effectors (31). In addition, activation of PRRs by commensal-derived signals promotes the production of antimicrobial peptides such as REGIII β and REGIII γ , reinforcing mucosal defense and maintaining intestinal homeostasis (104,115).

Commensal bacteria also regulate the development and function of innate immune cells. The microbiota promotes maturation of intestinal macrophages and dendritic cells (DCs) toward a tolerogenic yet responsive phenotype. For example, gut macrophages continuously exposed to microbial signals such as SCFAs acquire an anti-inflammatory profile characterized by high IL-10 production and reduced expression of pro-inflammatory cytokines like IL-6 and IL-12. This allows them to clear microbes and apoptotic cells efficiently while preventing excessive tissue damage (115,116). Similarly, gut DCs conditioned by commensals display reduced

inflammatory potential but retain strong antigen-sampling and regulatory capacity. CD103⁺ DCs migrate to mesenteric lymph nodes and promote the differentiation of regulatory T cells (Tregs) through retinoic acid and TGF- β signaling, thereby enforcing oral tolerance to dietary and microbial antigens. In contrast, CX3CR1⁺ DCs extend transepithelial dendrites to sample luminal bacteria and maintain barrier surveillance without triggering overt inflammation under homeostatic conditions (115). Importantly, this balance is maintained through microbiota-derived metabolites such as SCFAs, which epigenetically reprogram phagocytes by inhibiting histone deacetylases (HDACs) and activating G-protein–coupled receptors (117). In germ-free or dysbiotic states, this regulatory imprinting is lost, leading to impaired cytokine production, defective T-cell priming, and increased susceptibility to infection or autoimmunity (118).

2.6.1.2 Epithelial Barrier Integrity: Tight Junctions, Mucus, and Microbiota Influence

The intestinal epithelium functions as both a physical and immunological barrier. Commensal metabolites such as SCFAs including acetate, propionate, and butyrate, serve as key regulators of epithelial function. SCFAs bind G-protein-coupled receptors (GPCRs) (e.g., GPR43) and regulate immune cell differentiation and activation. For example, *Akkermansia muciniphila* and *Prevotella* spp are key acetate producers that activate GPR43-mediated signaling (119). SCFAs also support epithelial defenses by promoting of IL-22 production, which upregulated mucus secretion and increase barrier integrity (119). In addition, specific SCFAs have been shown to directly enhance mucin production and goblet cell differentiation. For instance, acetate has been demonstrated to upregulate transcription factors such as KLF4, promoting goblet cell differentiation and mucin synthesis in the colonic epithelium (120). Furthermore, studies using gnotobiotic models have shown that commensal bacteria such as *Bacteroides thetaiotaomicron* (an acetate producer) increase expression of mucus-related genes and stimulate goblet cell development, thereby enhancing mucin production and glycosylation patterns (121). Butyrate, a major SCFA produced by Firmicutes, has also been widely reported to stimulate mucin gene expression (e.g., MUC2) and reinforce the mucus layer, highlighting the role of microbial fermentation products in maintaining mucosal protection (122).

Beyond mucus production, the gut microbiota and their metabolites play a critical role in regulating tight junction (TJ) integrity. SCFAs such as acetate, propionate, and butyrate have

been shown to upregulate the expression of key TJ proteins, including occludin, claudins, and zonula occludens-1, thereby reducing paracellular permeability and strengthening barrier function (123). In parallel, commensal bacteria such as *Akkermansia muciniphila* contribute to barrier integrity by enhancing mucus layer thickness and supporting epithelial structure, which indirectly stabilizes TJ organization (124)

2.6.2 Role of Microbiota in Development of Adaptive Immunity

The gut microbiota also contributes to shaping the adaptive immune system by coordinating the development, activation, and regulation of lymphocytes and antibody-producing cells. The lamina propria houses a dense population of immune cells, including T cells, B cells, macrophages, and dendritic cells, which work together to maintain tolerance to harmless antigens while defending against pathogens (104). A key inductive site for mucosal adaptive responses is Peyer's patches, clusters of organized lymphoid follicles located mainly in the ileum of the small intestine (125). Luminal antigens are transported across the epithelium by M cells and captured by DCs, which process them and present microbial peptides on MHC class II molecules. MHC II-dependent antigen presentation is essential for activating naïve CD4⁺ T cells and guiding their differentiation into distinct lineages (126). The outcome of this differentiation depends on both the microbial signals and the local cytokine environment (127). This process also generates long-lived memory cells, enabling rapid responses upon antigen re-encounter. The trafficking and localization of lymphocytes are tightly regulated through adhesion molecules, a process known as homing, ensuring immune cells reach the appropriate sites in the gut. Disruption of these pathways can contribute to autoimmune or inflammatory disorders (31). Commensal microbes regulate this process through two main mechanisms: antigen presentation and microbial metabolites. First, DCs sensing commensals via PRRs modulate MHC II-dependent presentation together with co-stimulatory signals, thereby directing T cell fate (126). Second, microbiota-derived metabolites, especially SCFAs such as butyrate and propionate epigenetically reprogram T cells and DCs by inhibiting histone deacetylases and activating GPCRs, promoting Foxp3⁺ regulatory T cell (Treg) differentiation (128). Gut commensals also play a central role in maintaining immune homeostasis, defined as the dynamic balance between pro-inflammatory and anti-inflammatory immune responses that preserves tissue integrity while allowing effective defense against pathogens (129). In the

intestine, immune homeostasis requires controlled activation of effector T cells to combat infection while preventing excessive inflammation that may damage host tissues (129,130). Gut commensals also regulate the balance between anti-inflammatory and pro-inflammatory T-cell responses. For example, certain microbial taxa, such as *Clostridium* clusters IV and XIVa, promote the differentiation and expansion of Foxp3⁺ regulatory T cells (Tregs) via mechanisms involving SCFAs (110,131). Conversely, segmented filamentous bacteria (SFB) and similar organisms drive Th17 lineage differentiation, leading to IL-17-mediated mucosal defense, although excessive Th17 responses may contribute to inflammation. For example, *Bacteroides fragilis* uses polysaccharide A to signal through TLR2 on DCs, favoring Treg induction and suppression of excessive inflammation. In contrast, pathogens such as *Citrobacter rodentium* induce inflammatory Th17 responses (131,132).

2.6.3 Role of Microbiota in Colonization Resistance

Colonization resistance is the process by which the gut's microbial community prevents the colonization of ingested bacteria and controls the overgrowth of resident bacteria that are usually found in low numbers. This resistance applies to both commensal and pathogenic bacteria (133). There are two types of colonization resistance mechanisms: direct and indirect (134).

2.6.3.1 Direct Mechanisms of Microbial Colonization Resistance

Direct mechanism of colonization resistance occurs through the competition between bacteria. Bacteria compete with each other over resources and physical space or compete by creating inhibitory substances (22,135). Direct mechanisms include: spatial competition, nutritional competition, prokaryotic antimicrobial peptides, and inhibitory metabolites (134).

2.6.3.1.1 Spatial Competition

Bacteria compete not only for nutrients but also for ecological niches, which include defined physical locations (such as epithelial attachment sites or mucus layers) and specific metabolic resources within the gut. This compartmentalization of microbes into distinct niches limits how many organisms can occupy the same space and perform the same functions. As a result,

competition typically occurs between closely related species, because they rely on similar nutrients and attachment sites (136,137). Niche competition leads to competitive exclusion when two strains with similar ecological requirements attempt to colonize the same niche. In such cases, the strain that establishes first can occupy the available attachment sites and resources, preventing the second strain from persisting in that environment (138,139). Generally, species that have the greatest gene functions in common with one another compete directly with each other. For instance, species of *Citrobacter* or *Clostridium* can inhibit the colonization of their more harmful relatives(135,140,141).

2.6.3.1.2 Nutritional Competition

The gut microbiota is a stable community that has reached equilibrium and remains generally stable over time, except when disrupted by events like antibiotic use or other events that can change the bacterial composition of the gut (134). This stability is maintained because the populations of most resident intestinal bacteria are regulated by one or a few key nutrients that specific strains can utilize most effectively (142). By efficiently scavenging these nutrients, the gut microbes limit their availability, preventing the overgrowth of pathogens and maintaining a balanced microbial environment (134). For instance, *Bacteroides* strains possess the enzyme required to digest a complex polysaccharide that other bacterial strains lack (142). During gut inflammation, iron availability in the gut decreases as part of the nutritional immune response, which typically inhibits bacterial growth. However, *Salmonella enterica* serovar Typhimurium can survive and even thrive in this environment because it exploits inflammation-associated conditions: it utilizes alternative carbon and energy sources, resists host antimicrobial proteins and expresses specialized metal transport systems that allow efficient iron acquisition under iron-limited conditions (143).

2.6.3.1.3 Prokaryotic Antimicrobial Peptides

Antimicrobial molecules play a vital role in maintaining the microbiome stability by inhibiting major microbial competitors. These molecules are diverse in structure, and generated by bacteria, encoded by rapidly evolving biosynthetic gene clusters (BGCs) (144,145). These antimicrobials fall into two broad categories: (i) antimicrobial peptides, such as bacteriocins, which are ribosomally synthesized and often post-translationally modified (RiPPs) (144,145)

and (ii) larger secondary metabolites, including non-ribosomal peptides (NRPs) and polyketides (PKs), which are encoded by complex biosynthetic gene clusters (146). Both classes display bacteriostatic or bacteriocidal activity against pathogens and symbiotic bacteria (147). Bacteriocins often target closely related species and generally have a specific range of action. However, some, particularly those produced by gram-positive bacteria, can have a broader spectrum. Different classes of antimicrobial molecules encoded by BGCs will be discussed later under the section 2.8 “Microbial Derived Metabolites and Natural Products as Alternative Therapeutics.”

2.6.3.1.4 Other Metabolites with Antimicrobial Activities

Bacterial strains can produce byproducts that inhibit the growth of other gut microorganisms. For instance, bile acids, which are produced by the host to aid in the emulsification and absorption of dietary fats, are conjugated to taurine or glycine in the liver and then released into the small intestine (148,149). In the small intestine, certain bacteria, such as *Clostridium scindens*, modify these bile acids to produce secondary bile acids (148,150). These secondary bile acids, such as lithocholic acid and deoxycholic acid, have inhibitory effects on gram-positive bacteria, including the vegetative forms of *Clostridium difficile* (148).

SCFAs such as acetic, propionic, and butyric acid, are created in the large intestine by diverse lineages of obligate anaerobe bacteria, including *Bacteroides* and clostridia that are abundant in adult mammalian microbiota. SCFAs are mainly produced through fermentation of nondigestible carbohydrates. Intestinal epithelial cells utilize SCFAs as an energy source, but they can also contribute to colonization resistance (140,151). For instance, acetic and butyric acid prevent *Salmonella* species from colonizing the mouse gut and stop the growth of harmful *E. coli* and *C. difficile* (152,140,153).

2.6.3.2 Indirect Mechanism of Colonization Resistance

Apart from the direct prevention of pathogen colonization by symbionts or their byproducts, microbes compete each other through indirect defense mechanisms, immune responses, the mucus layer, oxygen gradients, and other mechanisms that the gut microbiota induces or maintains to prevent pathogen colonization among them (154).

2.6.3.2.1 The Mucosal Barrier

Microbial symbionts are essential in limiting pathogen colonization by reinforcing the mucosal barrier in the gut, particularly through the mucus layers in the small intestine, cecum, and colon (155,156). These mucus layers, mainly composed of the glycoprotein mucin 2 (*Muc2*), are crucial for excluding bacteria and other particles. The gut microbiota not only stimulates the production of these mucus layers but also releases compounds that affect pathogen colonization. For example, fucosylated proteins shed from intestinal epithelial cells can be metabolized by gut bacteria to release L-fucose, which reduces pathogen virulence and decreases susceptibility to infections like *Citrobacter rodentium* (157,158). Conversely, antibiotic treatments can disrupt the microbiota, leading to the release of sugars from the mucus layer that fuel the growth of harmful bacteria like *Salmonella enterica* serovar Typhimurium and *Clostridium difficile* (155,159). In addition, *Muc2* and its glycosylation, particularly α (1,2) fucosylation, play a key role in host-microbe interactions, serving as nutrient sources or receptors for microbes, providing attachment site for commensals, which once attached, prevent attachment of pathogens and reducing intestinal pathology caused by *C. rodentium* (142).

2.6.3.2.2 Oxygen Limitation

The gut microbiota stimulates alterations in the gut environment that can indirectly result in colonization resistance against pathobiont and enteric pathogens. Intestinal hypoxia, which is created and maintained by symbiotic bacteria to prevent the growth of harmful facultative anaerobes, is an obvious illustration of this impact (154). For example, symbiotic bacteria like clostridia species produce butyrate that promotes aerobic respiration in intestinal epithelial cells, thereby reducing oxygen levels at the epithelial surface (148,160). This hypoxic environment limits the growth of pathogenic facultative anaerobes, such as *Salmonella enterica* serovar Typhimurium. When clostridia are depleted such as in antibiotic therapy and consequently microbiota dysbiosis, oxygen levels increase, fueling the expansion of these pathogens (148,161).

2.6.3.2.3 Host-derived Antimicrobial Peptides and Proteins

The gut microbiota stimulates host epithelial cells to produce various host-derived antimicrobial peptides that target both symbionts and pathogens by disrupting bacterial membranes (148,162). Peptides like RegIII β and RegIII γ , produced by Paneth cells, help maintain spatial separation between bacterial symbionts and intestinal epithelial cells (154,163). A reduction in these host-derived peptides, often caused by antibiotic use, increases susceptibility to infections by enteric pathogens. Other antimicrobial peptides, like cryptdins, are induced by microbiota-derived signals, such as *NOD2* stimulation, and contribute to clearing pathogens like *Listeria monocytogenes* (148,164). Lipocalin 2 (LCN2) is another antimicrobial protein that prevents pathogen iron acquisition by sequestering bacterial siderophores. LCN2 production is induced by inflammation and depends on the gut microbiota (148,165). Mice lacking LCN2 show elevated levels of iron-scavenging bacteria, indicating its role in regulating microbiota composition (148,166).

2.6.3.2.4 Cytokines

A protective function for colonization resistance against enteric pathogens can be played by certain cytokines that are stimulated by the microbiota. For instance, IL-22 is one such cytokine, induced by bacteria like segmented filamentous bacteria (SFB), which increases resistance to infections such as *C. rodentium* by enhancing intestinal barrier function and altering gut microbiota composition (148,167). Other cytokines like IL-1 β and IL-36, also induced by the microbiota, help in pathogen clearance and resistance through various mechanisms. For example, IL-1 β enhances neutrophil recruitment to clear pathogens (148,168), and butyrate-producing bacteria limit pathogen colonization by regulating intestinal tight-junction proteins through IL-10 signaling (148,160).

2.7 Gut Microbiome and Untapped Potentials as Alternative Therapeutics

Microbial communities synthesize a diverse array of bioactive molecules commonly referred to as microbial natural products (MNPs). These compounds are produced by a wide range of microorganisms, including bacteria and fungi, and contribute to numerous biological functions within complex ecosystems (167). A substantial proportion of MNPs belong to the class of

secondary metabolites which are molecules that are not essential for basic cellular processes but instead provide adaptive advantages under specific environmental conditions. Rather than supporting growth directly, these metabolites facilitate ecological interactions by shaping microbial community structure through mechanisms such as chemical signaling, resource competition, and antimicrobial activity (33,34). At the genetic level, the biosynthesis of many of these compounds is directed by biosynthetic gene clusters, which are co-localized sets of genes encoding enzymes and regulatory elements required for specialized metabolite production (168–170).

Recent research has increasingly highlighted that the intestinal microbiota exerts much of its influence on host physiology through the production of MNPs, which act as critical mediators in host-microbe communication. Consequently, there is growing interest in metabolite-based therapeutics, which aim to modulate these downstream signaling pathways rather than directly altering microbial composition (171). Experimental studies in animal models have identified several promising candidates for metabolite-based interventions, including SCFAs, which exhibit anti-inflammatory properties and are altered in inflammatory bowel disease (171,172).

Therapeutic strategies targeting the microbiome can be broadly categorized into probiotic and postbiotic approaches. Probiotics are defined as live microorganisms that, when administered in adequate amounts, confer a health benefit on the host (173). In contrast, postbiotics refer to preparations of inanimate microorganisms and/or their components that confer a health benefit, including purified microbial metabolites, cell wall components, or secreted bioactive compounds (174). While probiotic therapies rely on administering live organisms with the expectation of engraftment or functional activity in situ, postbiotic strategies focus on delivering defined microbial-derived molecules that mediate specific biological effects (174).

Increasing interest has emerged in the therapeutic application of postbiotic agents, particularly because these compounds can offer significant advantages over live microbial therapies. Unlike probiotics, which require maintenance of viable cells throughout processing and storage and are subject to variability in live-to-dead ratios over shelf-life, postbiotic compounds exhibit superior physicochemical stability, often remaining stable for extended

periods at room temperature without reliance on cold-chain logistics (174). Their defined composition enables more consistent dosing, improved reproducibility, and clearer safety assessment, as concerns related to microbial replication, translocation, or rare cases of bacteraemia are minimized (174,175). However, challenges remain. Many microbiome-derived metabolites exhibit rapid systemic clearance, requiring sustained delivery strategies, and their biological effects may be context-dependent, varying across tissues or host physiological states. Despite these limitations, MNP-based therapeutics represent a promising alternative to whole-microbe approaches, offering a mechanistically targeted strategy to harness microbiome functionality (171).

2.8 Microbial Derived Metabolites and Natural Products as an Alternative to Antibiotics

MNPs and other microbial metabolites function as chemical mediators that regulate competition, cooperation, and spatial organization within microbial consortia, as well as host–microbe interactions (176). For example, bacteriocins produced by commensal *Escherichia coli* and *Lactobacillus spp.* can inhibit closely related enteric pathogens, thereby contributing to colonization resistance (177). SCFAs, which are another group of MNPs including primarily acetate, propionate, and butyrate, are produced via microbial fermentation of indigestible carbohydrates by commensal taxa such as *Faecalibacterium prausnitzii*, *Roseburia intestinalis*, and *Anaerostipes butyraticus* (178,179). These metabolites play a central role in maintaining intestinal homeostasis by supporting epithelial repair, promoting cellular proliferation and differentiation, and strengthening barrier integrity. Acetate, largely produced by *Bifidobacterium spp.*, enhances epithelial barrier function and modulates inflammation through activation of G-protein–coupled receptor 43 (GPR43) (144,180). GPR43 signaling also promotes microbiota-reactive IgA production and increases IgA coating of intestinal bacteria, including members of the Enterobacterales (180).

2.8.1 Secondary Metabolites

Secondary metabolites are chemically diverse compounds produced by microorganisms that are not essential for primary metabolic processes but play important roles in ecological

interactions (33,34). Secondary metabolites can be categorized into several classes; some of the most important classes are (36):

2.8.1.1 Ribosomally Synthesized and Post Translationally Modified Peptides (RiPPs)

These metabolites are often produced by bacteria to help them compete with one another. Typically, these substances target bacterial strains that are closely related to the bacteria that produced them, though, some could also show broader activity (145). RiPPs include five main subclasses (181,182).

2.8.1.1.1 Bacteriocins

Bacteriocins are peptides synthesized by ribosomes that exhibit bactericidal properties (36). They can target a wide range of bacterial species (broad-spectrum) or be selective to specific ones (narrow-spectrum) (183). Both gram-positive and gram-negative bacteria are capable of producing bacteriocins (183). Bacteriocins operate through a wide range of antimicrobial mechanisms. Their effects can include breaking down essential cellular components, disrupting or permeabilizing bacterial membranes, interfering with key enzymatic processes, and exerting other targeted actions that ultimately compromise the survival of susceptible bacteria (147). A notable characteristic of bacteriocins is that bacterial resistance to them is relatively uncommon, as it typically requires alterations in membrane composition or receptor structures that can incur significant fitness costs (183). Bacteriocins can also act as regulatory agents within microbial communities, shaping community structure by conferring a competitive advantage to beneficial bacteria over potential pathogens (183). Bacteriocins are commonly classified into major groups based on structure, size, and post-translational modification. Class I bacteriocins (lantibiotics) are small (<5 kDa), heat-stable peptides that undergo extensive post-translational modification. A well-characterized example is nisin, produced by *Lactococcus lactis*, which disrupts membrane integrity and inhibits peptidoglycan synthesis (145,182,184). Class II bacteriocins are small, heat-stable, unmodified or cyclic peptides that typically form pores in target membranes; examples include pediocin PA-1 and various enterocins. Gassericin T is another representative of class II produced by *Lactobacillus gasseri* SBT 2055 (145,182). Class III bacteriocins are larger (>30 kDa), heat-labile proteins with enzymatic or membrane-targeting activity, such as colicins produced by *Escherichia coli*, which can exhibit DNase,

RNase, or pore-forming functions (145,182,185). Despite their promising antimicrobial activity, the therapeutic application of bacteriocins faces several challenges. These peptides are often susceptible to proteolytic degradation in the gastrointestinal tract, which may limit their stability and bioavailability (186–188). Additionally, their relatively narrow antimicrobial spectrum and potential immunogenicity may restrict their effectiveness in complex microbial ecosystems such as the gut microbiota (189,190).

2.8.1.1.2 Lantibiotics

Lantibiotics are a specialized group of ribosomally synthesized antimicrobial peptides distinguished by their unusual amino acid composition and extensive post-translational modification. After translation, their precursor peptides undergo enzymatic dehydration of serine and threonine residues, followed by intramolecular addition of cysteines to form the characteristic lanthionine and methyllanthionine thioether rings. These intricate structural features create compact, highly stable molecules with potent antibacterial activity. Lantibiotic biosynthesis is typically encoded within dedicated gene clusters that include modification enzymes, transport proteins, regulatory elements, and immunity factors that protect the producer organism. Their mode of action often involves binding to lipid II, a key precursor in peptidoglycan synthesis, which both sequesters this essential molecule and promotes pore formation in the bacterial membrane (183,191,192). Lantibiotics are categorized mainly into two types based on their structure and mechanism of action. Type A lantibiotics are positively charged peptides that primarily disrupt bacterial cell wall biosynthesis and forming pores, leading to ion leakage and cell death; such as epidermin, produced by *Staphylococcus epidermis* (191). In contrast, Type B lantibiotics have a more compact, globular structure and inhibit bacterial growth mainly by interfering with cell wall synthesis, often through binding to lipid II (191,193). These small peptides primarily produced by Bacillota bacteria, and they target a narrow range of Gram-positive bacteria (36).

2.8.1.1.3 Microcins

Microcins are narrow-spectrum antibacterials, characterized by a variety of unique post-translational modifications, such as the transformation of cysteine and serine residues into

thiazoles and oxazoles (as seen in microcin B17), these are produced solely by enterobacteria, particularly *Escherichia coli* (181,194). These antibacterials are effective primarily against bacterial strains related to their producers (181,195). For instance, Microcin J25, which is produced by commensal *E. coli* strains, exerts its antimicrobial effects by inhibiting RNA polymerase in gram-negative bacteria and disrupting cell membranes in *Salmonella* and *E. coli* strains that overexpress the ferrichrome outer membrane transporter (fhuA receptor) (184,196). The fhuA is a TonB-dependent outer membrane transporter that serves as the specific recognition and translocation site for Microcin J25 in susceptible gram-negative bacteria (195,196).

2.8.1.1.4 Thiopeptides

Thiopeptides, also referred to as thiazolyl peptides, represent a distinctive class of RiPPs characterized by highly complex and densely modified macrocyclic structures. These natural products are predominantly produced by gram-positive bacteria and are notable for their potent antimicrobial activity, particularly against antibiotic-resistant gram-positive pathogens. Genome mining analyses revealed that thiopeptide BGCs are widely distributed among Bacillota and Actinomycetota, suggesting an important ecological role in microbial competition (197). Notably, lactocillin, a thiocillin-like thiopeptide produced by the vaginal commensal *Lactobacillus gasseri*, displays selective activity against vaginal pathogens while sparing beneficial microbiota, indicating a role in maintaining vaginal microbial homeostasis (197). Similarly, cutimycin, a berninamycin-like thiopeptide synthesized by the skin commensal *Cutibacterium acnes*, has been shown to limit colonization by pathogenic *Staphylococcus* species (197,198). Thiopeptides biological activity is largely attributed to specific interactions with essential components of the bacterial translation machinery, such as the ribosome or elongation factors, which results in effective inhibition of protein synthesis. Although thiopeptides were discovered several decades ago, their therapeutic development was historically limited by challenges related to poor solubility and pharmacokinetic properties (197,199).

2.8.1.2 Terpenoids

Microbiota-derived terpenoids are secondary bile acids produced from cholic acid (CA) and chenodeoxycholic acid (CDCA), which are synthesized in the liver. While most bile acids are reabsorbed, some reach the large intestine, where gut bacteria like *Clostridium scindens* convert them into toxic compounds, such as deoxycholic acid (DCA) and lithocholic acid (LCA). These secondary bile acids are linked to diseases like liver toxicity and colon cancer (181,200,201). At physiological concentrations, secondary bile acids exert beneficial effects, including modulation of host immunity (maintenance intestinal barrier function) and endocrine functions and energy homeostasis through activation of bile acid receptors such as farnesoid x receptor (FXR) and plasma membrane-bile acid receptor TGR5 (202,203). Furthermore, bile acids inhibit enteric pathogens through their detergent-like antimicrobial activity and via FXR-mediated induction of genes encoding antimicrobial peptides (203,204). While bile acid metabolites contribute to host defense and microbial regulation, their biological activity is highly concentration dependent. Excessive accumulation or chronic exposure to elevated levels of DCA and LCA has been associated with hepatotoxicity, DNA damage, oxidative stress, and promotion of colorectal carcinogenesis (205,206).

2.8.1.3 Polyketides (PKs)

PKs constitute one of the most widespread and structurally diverse classes of natural products encoded by BGCs in environmental and host-associated microbiomes (207). Polyketides are synthesized by large, multidomain enzyme complexes known as polyketide synthases (PKSs), which catalyze the sequential decarboxylative condensation of simple acyl-CoA building blocks (e.g., malonyl-CoA and methylmalonyl-CoA) in a process mechanistically analogous to fatty acid biosynthesis (207). Polyketides are classified into three major types based on their enzymatic organization and catalytic strategy. Type I PKSs are large, multifunctional polypeptides containing multiple catalytic domains that operate either modularly or iteratively (207). Type II PKSs consist of discrete, iteratively acting enzymes organized around a minimal PKS (KS α , KS β , and ACP) that synthesizes reactive poly- β -ketone intermediates, which are subsequently cyclized and aromatized to form polycyclic aromatic compounds (207,208). In contrast, Type III PKSs are simpler homodimeric ketosynthase-like enzymes that directly utilize acyl-CoA substrates and catalyze iterative chain elongation and cyclization within a single active site, generating structurally diverse aromatic scaffolds

(207,209). Clinically, polyketides have given rise to several major therapeutic agents, including antibiotics, antifungals, and anticancer drugs. For example, erythromycin, produced by *Streptomyces erythreus*, and tetracycline, derived from *Streptomyces rimosus*, are widely used antibacterial polyketides (167). Beyond antibacterials, polyketides include immunosuppressants such as tacrolimus and anticancer agents such as doxorubicin, underscoring their pharmaceutical significance. In addition to beneficial compounds, certain pathogen-associated PKs contribute to disease. For example, cereulide, produced by *Bacillus cereus*, disrupts mitochondrial function, leading to food poisoning (181,210). Mycolactones, produced by *Mycobacterium ulcerans*, cause the tissue necrosis seen in Buruli ulcer (181,211). Despite their major contributions to drug discovery, the development of polyketide-derived therapeutics can be challenging due to the structural and biosynthetic complexity of PKSs pathways, which complicates pathway engineering and scalable production (167).

2.8.1.4 Nonribosomal Peptides (NRPs)

NRPs constitute a large and structurally diverse class of bioactive secondary metabolites produced primarily by soil-inhabiting microorganisms, such as Actinomycetes and Bacilli, and eukaryotic filamentous fungi, marine microorganisms (210,211). Unlike ribosomally synthesized peptides, NRPs are assembled by large, multimodular enzyme complexes known as nonribosomal peptide synthetases (NRPSs), which catalyze the sequential condensation of diverse monomeric substrates during secondary metabolism. Importantly, NRP biosynthesis is not restricted to the 20 proteinogenic amino acids; instead, more than 500 distinct building blocks including nonproteinogenic amino acids, fatty acids, and α -hydroxy acids, can be incorporated, contributing substantially to their structural complexity and biological activity (211). They include several clinically important therapeutics, such as daptomycin (last-resort antibiotic), bleomycin (antitumor agent), and cyclosporin (immunosuppressant) (211,212). A prominent example is vancomycin, a glycopeptide antibiotic produced by *Amycolatopsis orientalis*, which remains essential for the treatment of methicillin-resistant *Staphylococcus aureus* and other gram-positive infections (213). Other medically significant NRPs include daptomycin from *Streptomyces roseosporus* (214). However, the therapeutic application of NRPs may also present limitations. Many NRPs exhibit complex structures that complicate large-scale synthesis and production (211,215).

2.8.2 Primary Metabolites

Primary metabolites are essential compounds involved in central cellular processes such as energy production, biosynthesis, and cellular maintenance, including amino acids, SCFAs, and organic acids. Although primarily associated with microbial growth and survival, increasing evidence indicates that these metabolites also influence microbial community dynamics. For example, fermentation-derived products such as short-chain fatty acids can modulate environmental pH, inhibit pathogen growth, and function as signaling molecules affecting both microbial and host pathways (29,216). Similarly, lactic acid and hydrogen peroxide produced by commensal bacteria contribute to antimicrobial activity and niche competition within microbial ecosystems (217).

2.8.2.1 Amino Acid Metabolites

Gut microorganisms, living in an anaerobic environment, use amino acids as electron acceptors for fermentation or electron transport. This process results in the production of metabolites that play vital roles in host physiology (36). For instance, indole, a metabolite of tryptophan, serves as a signaling molecule within bacterial communities (36,218). It also regulates immunity and colonization resistance against *Salmonella enterica* and enterohemorrhagic *E. coli* (219,220). Another tryptophan metabolite, tryptamine, acts as a neurotransmitter (36,37).

2.8.2.2 Oligosaccharides

Oligosaccharides can modulate the immune system by binding to specific ligand-receptor interactions. For instance, *Bacteroides*, the most prevalent bacterial genus in the human gut, produces various capsular polysaccharides (36). One of these, polysaccharide A from *Bacteroides fragilis* (38), interacts with the host's immune system via Toll-like receptor 2 (TLR2), stimulating regulatory T cells to release interleukin-10 (IL-10). This response helps regulate T helper 17 cell activity, facilitating *B. fragilis* colonization while also suppressing colitis caused by *Helicobacter hepaticus* (181,221,222).

2.8.2.3 Short Chain Fatty Acids

The gut microbiota is the primary source of SCFAs, which are produced through the fermentation of non-digestible polysaccharides such as dietary fibers and resistant starches. SCFA production and the composition of the gut microbiota change throughout life, influenced by diet and developmental stage. In early life, the gut microbiota of piglets is strongly influenced by a milk-based diet and is typically dominated by genera such as *Bacteroides*, *Lactobacillus* and *Fusobacterium*, which are adapted to metabolize milk-derived carbohydrates and host-derived glycans (221). These early colonizers primarily produce metabolites such as lactate and other intermediate fermentation products. However, the transition from milk to a solid, cereal-based diet at weaning induces substantial shifts in the composition and metabolic capacity of the gut microbiota (222). This transition promotes the enrichment of fiber-degrading and SCFA-producing bacteria, particularly members of the families Lachnospiraceae and Ruminococcaceae (222). Consequently, genera such as *Roseburia*, *Ruminococcus*, *Lachnospira*, *Coprococcus*, *Prevotella* and *Dorea* increase in relative abundance (222,223). These taxa possess metabolic pathways enabling the degradation of complex polysaccharides and resistant starch, leading to the production of SCFAs such as acetate, propionate, and butyrate (222,224). Among SCFAs, butyrate has the greatest physiological impact, providing energy for colonocytes, maintaining an anaerobic gut environment, and supporting epithelial barrier function. Key butyrate-producing bacteria include members of the Lachnospiraceae (e.g., *Roseburia*, *Blautia* and *Eubacterium hallii*) and Ruminococcaceae and Oscillospiraceae (e.g., *Faecalibacterium prausnitzii*) families, which also participate in metabolic cross-feeding with acetate and propionate producers. Thus, the post-weaning expansion of SCFA-producing bacteria reflects the functional maturation and dietary adaptation of the piglet gut microbiota (222,225). SCFAs play central roles in maintaining gut health through multiple mechanistic pathways. Butyrate serve as key signaling metabolites that enhance epithelial barrier integrity by upregulating the expression and assembly of tight junction proteins such as occludin, claudins, and zonula occludens-1 (ZO-1), thereby reducing paracellular permeability and preventing bacterial translocation (123,226,227). SCFAs also promote the mucus layer by increasing the expression of mucin genes (e.g., MUC2) in goblet cells, contributing to a thicker and more protective mucus gel that limits microbial contact with the epithelium (226,228). Additionally, SCFAs modulate host-microbe interactions by regulating antimicrobial peptide

production, particularly through effects on Paneth cells; butyrate enhances the expression of defensins and other AMPs by activating G-protein–coupled receptors (e.g., GPR43) and by serving as a histone deacetylase inhibitor, which influences gene transcription in Paneth cells and intestinal epithelial progenitors (226,229).

2.9 Clinical Relevance and Therapeutic Potential of Microbiota-Derived Secondary Metabolites

Microbiota-derived secondary metabolites have attracted increasing interest as antimicrobial alternatives and functional modulators of gut microbial ecosystems, particularly in the context of reducing antibiotic use in livestock production (181,230). Among these compounds, bacteriocins represent the most advanced group in terms of practical application. The lantibiotic Nisin, produced by *Lactococcus lactis*, is widely used as a food preservative and has been extensively studied for controlling gram-positive pathogens such as *Listeria monocytogenes*, *Clostridium perfringens*, and *Staphylococcus aureus* in food systems and animal production environments (145,231). Similarly, enterocin produced by *Enterococcus species* and pediocin-like bacteriocins have demonstrated inhibitory activity against enteric pathogens including *Salmonella* spp. and pathogenic *Escherichia coli*, supporting their potential application for controlling gastrointestinal infections in livestock (230,232). In swine and poultry production systems, bacteriocins can be administered either as purified antimicrobial peptides incorporated into feed additives or through probiotic strains capable of producing bacteriocins *in situ* within the gastrointestinal tract, thereby suppressing pathogenic bacteria while maintaining beneficial members of the gut microbiota (145,230,233).

Experimental studies further demonstrate that bacteriocins or bacteriocin-producing bacteria can influence intestinal microbial communities and suppress pathogens *in vivo* (145). For example, administration of *Lactococcus lactis* strains to human-flora-associated rats increased fecal Bifidobacterium populations while reducing enterococci and streptococci in several intestinal compartments (232,234). In another study, the commensal *Escherichia coli* strain H22, which produces multiple bacteriocins including colicin Ib, colicin E1, and microcin C7, eliminated *Shigella flexneri* from the intestinal tract of germ-free mice following oral inoculation (232,235). Similarly, the probiotic strain *Lactobacillus salivarius* UCC118

produces the bacteriocin Abp118, which was shown to protect mice from infection with *Listeria monocytogenes*, whereas a mutant strain lacking bacteriocin production failed to confer protection (232,236). Importantly, similar effects have been observed in swine models. Dietary supplementation with bacteriocin-producing lactic acid bacteria, including *Lactobacillus salivarius* and *Pediococcus Pentosaceus*, has been shown to reduce intestinal colonization by *Salmonella enterica* and improve gut microbial balance in weaned pigs (237,238). Additionally, bacteriocin-producing probiotics have been associated with improved growth performance and reduced post-weaning diarrhea (9).

Other classes of microbiota-derived secondary metabolites have also yielded clinically important antimicrobial compounds. For example, several polyketide antibiotics, including erythromycin and tetracycline, originate from microbial secondary metabolism and remain widely used antibacterial agents (167). Likewise, NRPs have produced therapeutically important antibiotics such as vancomycin and daptomycin, which are essential for the treatment of multidrug-resistant Gram-positive infections (211). In addition to direct antimicrobial activity, certain microbiota-derived metabolites contribute to host health through modulation of intestinal physiology and immunity. For instance, secondary bile acids generated by gut microbes can regulate intestinal barrier integrity and antimicrobial peptide expression through host bile acid receptors (203,239,240).

2.10 Host Specificity of Microbiome and Evolutionary Relevance

Emerging evidence indicates that host specificity is a critical determinant of the ecological function and applied potential of microbial metabolites and probiotic strains, as microbes and their bioactive products are shaped by long-term co-evolution with their animal hosts (241,242). Support for host adaptation is provided by experimental studies: rat-specific strains of *Lactobacillus reuteri* efficiently colonize the mouse gut, whereas human-specific strains fail to do so (242,243); similarly, bacterial commensals originating from honey bees are unable to colonize bumble bees (242,244). Host-associated microbiomes often harbor unique and novel BGCs not found in environmental or non-host microbiomes, highlighting the potential for discovering host-adapted secondary metabolites with specific functional roles. For example, comprehensive genome mining of the human gut microbiome revealed thousands of diverse

BGCs families with unexplored biosynthetic potential (245,246). Similar analysis of the porcine gut microbiome identified a rich repertoire of secondary metabolite gene clusters, such as arylpolyene, resorcinol, NRPS, and bacteriocin clusters, with distinct distributions across intestinal compartments (247). Metagenomic surveys further indicate that many of these BGCs are uncharacterized and largely distinct from those found in humans and other animals, highlighting a reservoir of host-adapted bioactive compounds uniquely suited to the porcine gastrointestinal environment (247,248).

2.11 Genetic Evolution of Microbes to Acquire Traits Providing Niche Advantage

Microbial adaptation to ecological niches is driven in part by the evolution and diversification of biosynthetic gene clusters and other accessory metabolic pathways that encode specialized metabolic functions conferring competitive advantage (249,250). The evolution of BGCs can be shaped by both adaptive and neutral evolution (250). Adaptive evolution occurs when beneficial mutations, gene duplications, or horizontal gene transfer (HGT) events increase microbial fitness under selective pressures, such as inter microbial competition or host-specific interactions (250). In contrast, neutral evolution can allow BGCs to accumulate in genomes through genetic drift, periods of weak selection, or conditional activation, especially in actinomycetes, where many clusters remain silent under standard conditions (250,251). BGC formation and diversification often involve gene duplication, sequence divergence, and recombination, enabling microbes to explore novel metabolic pathways while retaining functional redundancy (252). Moreover, BGCs can be mobilized via plasmids or other mobile genetic elements, particularly in *Streptomyces* and related taxa, facilitating horizontal transfer of biosynthetic traits, though retention is influenced by fitness costs and ecological relevance (250,253). It is important to note that not all secondary metabolites are encoded within classical BGCs. Small antimicrobial peptides such as many bacteriocins and host-associated peptides are often not accompanied by large multienzyme assembly systems, regulatory operons, or transporter genes typical of PKS/NRPS BGC architectures, and therefore require alternative identification methods that do not rely on gene cluster detection (253). Bioinformatic tools for BGC mining (e.g., antiSMASH, PRISM) leverage signatures of core biosynthetic enzymes and conserved domain architectures to predict cluster boundaries, but are not optimized for small, standalone AMP genes, which may be

detected using specialized approaches such as hidden Markov model (HMM) profiles of AMP families or machine-learning classifiers trained on AMP physicochemical features (254,255).

2.12 Methods for Studying the Functional Potential of the Gut Microbiome

Over the past decade, culture-independent approaches, particularly 16S rRNA gene amplicon and shotgun metagenomic sequencing have provided important insights into the taxonomic composition and functional potential of the swine gut microbiome (256,257). These studies have revealed a core set of microbial taxa and metabolic pathways associated with host performance and health status (258). However, much of this knowledge remains correlative, as the lack of high-quality, genome-sequenced isolates constrains efforts to assign functions to specific microbes, validate predictions, or translate findings into actionable interventions. Exploring the microbiome's functional potential at the gene and genome level can provide insights into metabolic pathways and BGCs that influence microbial ecology and host interactions (259). The two main approaches for this are metagenomics and culturomics:

2.12.1 Shotgun Metagenomics:

Shotgun metagenomic sequencing provides a comprehensive approach to studying microbial communities by capturing all DNA present in a sample rather than targeting specific marker genes such as 16S rRNA (260,261). This untargeted strategy enables the detection of bacteria, archaea, fungi, and DNA viruses, and allows simultaneous characterization of both the taxonomic composition and functional potential of microbial communities through the identification of gene-coding regions (260,262). Metagenomic reads can be assembled either *de novo*, without relying on reference genomes, or by reference-guided approaches that map reads to known genomes (260). De novo methods are supported by tools like MEGAHIT, metaSPAdes, and IDBA-UD. Alternatively, read-based profiling approaches classify sequences directly against reference datasets for taxonomic or functional annotation using tools such as Kraken and MetaPhlAn, which rely on k-mer patterns or clade-specific marker genes (260,263,264).

A major advancement in metagenomic analysis is the reconstruction of metagenome-assembled genomes (MAGs), which are draft microbial genomes recovered directly from metagenomic sequencing data through binning of assembled contigs based on sequence composition, coverage, and co-abundance patterns (265,266). MAGs enable the recovery of genomes from uncultured or previously unknown microorganisms, significantly expanding our understanding of microbial diversity and metabolic potential in complex environments such as the gut microbiome (267). Importantly, MAG-based analyses enables the linkage of functional genes, metabolic pathways, and biosynthetic gene clusters to specific microbial taxa, facilitating the discovery of novel microbial metabolites and ecological functions (146). Despite these advantages, MAGs also have limitations. Genome reconstructions are often fragmented or incomplete, and errors such as contamination or mis-binning can occur when closely related organisms share similar genomic features (265,268,269). Additionally, metagenomic assemblies may fail to recover low-abundance organisms or genomic regions containing repetitive sequences, which can lead to incomplete representation of metabolic pathways or BGCs (270). Because of these limitations, metagenomic approaches are increasingly complemented by culturomics, which involves high-throughput cultivation strategies to isolate live microbes from complex communities. Culturomics enables the recovery of high-quality complete genomes and experimentally tractable isolates, facilitating functional characterization and validation of metabolic capabilities predicted from metagenomic analyses (271).

2.12.2 Culturomics:

Culturomics represents a high-throughput, culture-based approach aimed at significantly expanding the repertoire of microbial species that can be isolated from complex ecosystems (272). Unlike traditional microbiology, which relies on a limited number of culture conditions, culturomics systematically varies multiple parameters including culture media composition, incubation time, temperature, and atmospheric conditions (aerobic, anaerobic, or microaerophilic) to promote the growth of a broad range of microbial taxa (272,273). Importantly, culturomics provides access to isolate genomes, which are generally of higher quality than MAGs. MAGs are reconstructed from mixed-community sequencing data and are therefore susceptible to assembly artifacts, including chimeric sequences and contamination arising from closely related strains or species. In contrast, genomes derived from cultured

isolates originate from a single organism, resulting in more accurate, less fragmented assemblies. Even draft isolate genomes which are composed of multiple contigs, typically exhibit higher completeness and lower contamination than MAGs, while complete genomes (closed, circular sequences) provide the highest level of genomic resolution (274). Recent studies have highlighted that combining extensive culturing with rapid identification tools, such as matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and whole-genome sequencing, has transformed our understanding of gut microbial diversity (275). Metagenomic approaches, while powerful for detecting DNA signatures, often fail to capture rare, low-abundance, slow-growing, or highly oxygen-sensitive microorganisms. By exploiting hundreds of culture conditions, culturomics can isolate these overlooked species, enabling the identification of novel bacterial taxa (273). The availability of living cultures also allows researchers to generate high-quality reference genomes, which improve the accuracy of downstream sequencing analyses and facilitate the reconstruction of microbial metabolic networks (273,276). A key advantage of culturomics is that it provides live microbial isolates, enabling experimental characterization of microbial physiology, metabolism, antimicrobial resistance, and host–microbe interactions. These isolates can also be screened for the production of biologically active compounds such as secondary metabolites, SCFAs, anti-inflammatory molecules, and enzymes with pharmaceutical potential (247). Despite these advantages, culturomics also presents several limitations. Large-scale culturing experiments require substantial time, labor, and specialized infrastructure, including diverse culture media and anaerobic systems (277).

2.13 Use of Bioinformatic and Chemogenomic tools to Assess Therapeutic Potential of Microbiomes

Advances in sequencing and culturomics have shifted microbiome research from descriptive surveys to functional discovery of microbe-derived molecules with therapeutic potential. The integration of cultivation and genomic analysis provides access to live isolates and high-quality genomes, thereby enabling the experimental identification of bioactive compounds and host-modulating metabolites (277). In human studies, systematic genome mining of skin, gut, and other body sites has identified hundreds of RIPPs and natural products with antimicrobial

activity against pathogens including methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant Enterococci (278,279). Similarly, novel microbiome-derived peptides like lactomodulin from *Lactobacillus rhamnosus* have been identified through genome mining approaches combined by functional screening, and have been shown to exhibit both antimicrobial and anti-inflammatory effects *in vitro* (280). Moreover, Microcin J25 which is a lasso peptide from human faecal microbiota was identified through classical culture-based isolation followed by molecular and genomic characterization, and demonstrates potent activity against Gram-negative bacteria (279,281). Such discoveries illustrate how microbiome functional genomics can lead not only to new antimicrobials but also to modulators of host immunity and microbiota composition. Integrating these tools with culturomics bridges the gap between theoretical microbiome studies and translational applications by enabling identification and validation of candidate probiotics, postbiotics, and natural products with health benefits (276). Systematically uncovering this chemical diversity requires robust computational tools. Genome mining platforms such as antiSMASH enable systematic scanning of microbial genomes for biosynthetic gene clusters, thereby linking functional insights from *in vitro* studies to genomic predictions of novel secondary metabolites (257). antiSMASH is designed to systematically identify, annotate, and analyze BGCs involved in the production of specialized metabolites such as NRPs, PKs, bacteriocins, terpenes, and other bioactive compounds (253). The core functionality of antiSMASH relies on recognizing conserved biosynthetic domains and gene organization patterns characteristic of known BGC classes (47). It compares genomic regions against curated databases of experimentally characterized clusters and predicts cluster boundaries, biosynthetic types, and putative product classes. In addition to identifying well-established BGC families, antiSMASH is capable of detecting hybrid clusters and divergent or previously uncharacterized gene assemblies, thereby facilitating the discovery of novel secondary metabolite pathways (47,282,283). In addition to BGC-focused platforms, specialized bioinformatic tools have been developed for the discovery of AMPs directly from genomic and metagenomic data. For example, Macrel employs machine learning algorithms combined with sequence-derived features to identify and classify putative AMPs, enabling high-throughput screening of short peptide sequences with antimicrobial potential (284). Moreover, gutSMASH is a computational tool that systematically identifies primary metabolic gene clusters (PMGCs) in genomes derived from gut microbiota, addressing

a major limitation of conventional genome annotation pipelines that largely overlook clustered primary metabolism (285,286). Built upon the antiSMASH framework, gutSMASH is specifically adapted to detect conserved primary metabolic pathways characteristic of gut-associated microbes (286). Application of gutSMASH facilitates large-scale mapping of metabolic capabilities across gut microbial genomes, revealing extensive diversity in pathways involved in carbohydrate utilization, amino acid metabolism, bile acid modification, and other host-relevant biochemical processes (181,285). Complementing these approaches, CAZymes can be annotated using dbCAN3 (v5.0.6), which integrates multiple computational tools to enable accurate identification and functional classification of enzymes involved in carbohydrate metabolism, providing insights into microbial polysaccharide utilization and nutrient acquisition (287).

2.14 Conclusion

Although substantial progress has been made in characterizing the swine gut microbiome through both culture-independent and culture-based approaches, the availability of high-quality reference genomes and cultured isolates remains limited relative to the taxonomic and functional diversity of the pig gastrointestinal tract (276,288). Early large-scale metagenomic studies substantially expanded genome catalogs by reconstructing thousands of MAGs, providing valuable insights into microbial diversity and metabolic potential. However, MAGs often lack complete genomes, cannot always resolve strain-level diversity, and are susceptible to assembly errors and chimeric sequences, limiting their utility for functional validation and experimental characterization (51,276,288,289).

Culture-based efforts have begun to address these limitations by establishing collections of cultivated swine gut bacteria. Nevertheless, these collections remain relatively small and geographically biased, frequently originating from a limited number of healthy animals or specific production systems, and therefore capture only a fraction of the microbial diversity present across different pig populations (276). Consequently, many phylogenetically and functionally important taxa remain uncultured or lack high-quality reference genomes, restricting comparative genomic analyses and experimental validation.

These limitations have important downstream consequences. The incomplete representation of cultured swine gut microorganisms constrains systematic exploration of BGCs, CAZymes, AMPs, and other functional traits that may contribute to host health. As a result, the discovery of novel probiotics, postbiotics, and microbiome-derived bioactive compounds remains slower than expected despite rapid advances in sequencing technologies. Expanding comprehensive, high-quality genome collections linked to cultured isolates therefore represents a critical priority for improving genome annotation, enabling functional validation, and accelerating the translation of microbiome research into sustainable interventions for pig health and production. The present study addresses this gap by expanding the available cultured genome repertoire and providing functional genomic characterization of previously underrepresented swine gut bacteria.

2.15 Hypotheses

1. Different lineages of the gut microbiota carry metabolic (MGCs) and biosynthetic gene clusters (BGCs) that are conserved across healthy pig populations indicating existence of a core MGCs and BGCs in the healthy swine gut microbiome
2. The distribution of the identified MGCs and BGCs varies in the gut microbiome of healthy vs. diseased pig cohorts (post-weaning diarrhea) indicating novel microbial biomarkers of health and disease in pigs

2.16 Objectives

- Create a comprehensive biobank and genome database of commensal gut bacteria isolated from clinically healthy pigs
- Determine the metabolic potential of swine gut microbiota via functional annotation of the genome of commensal bacteria for identification of conserved and novel BGCs and metabolic gene clusters (MGCs – including SCFAs and CAZymes)
- Identify microbial biomarkers of health and susceptibility to post-weaning diarrhea by metagenomic profiling of the distribution of BGCs and MGCs across independent healthy and diseased pig cohorts.

3. Chapter 3: Research Manuscript

Running Title: Genome-Resolved Profiling of an Expanded Swine Gut Isolate Collection Reveals Functional Signatures of Health and Disease

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3.1 Abstract

Background

High-resolution genome data are critical for understanding the functional roles of swine gut microbes. However, many dominant swine gut lineages remain uncultured or underrepresented in reference databases, limiting our ability to link microbial functions to host traits. Culture-independent metagenomics, while powerful for community profiling, often lacks the resolution required to assign functional potential to specific taxa, constraining efforts to translate microbiome associations into actionable insights. This limitation is particularly consequential for post-weaning diarrhea, a major challenge in swine production that results in substantial economic losses and increased antimicrobial use. Understanding the functional capacity of gut microbes and identifying health-associated microbial features is therefore critical for the development of microbiome-based interventions to promote gut health and reduce disease susceptibility.

Results

We constructed an integrated genomic resource of the swine gut and fecal microbiota comprising 300 high-quality, non-redundant bacterial isolate genomes recovered from pig gastrointestinal tract. The isolate collection encompasses 33 bacterial families, featuring several previously uncultured representatives of the *Lachnospiraceae*, *Lactobacillaceae*, *Desulfovibrionaceae*, *Clostridiaceae*, *Streptococcaceae* and other phylogenetically diverse lineages. Functional profiling of the isolate genomes revealed extensive diversity among both known and previously uncharacterized metabolic gene clusters, biosynthetic gene clusters (BGCs), and antimicrobial peptides (AMPs). To assess the ecological and clinical relevance of these features, we analyzed their distribution across the microbiota of pigs with and without post-weaning diarrhea. Metagenomic read recruitment from independent swine cohorts against the isolate genome database showed that key functional pathways, particularly BGCs and AMPs, robustly discriminated between healthy pigs and those with post-weaning diarrhea, with significantly reduced abundance in diarrhea-associated hosts. Several lineages, including

Aneurinibacillus, Blautia, Dorea, Prevotella, *Paratractidigestivibacter*, and *Parabacteroides* emerged as dominant contributors to health-associated functional traits.

Conclusion

Combining high-throughput culturomics with machine learning-based functional inference, this study establishes an expanded swine gut isolate genome resource and identifies microbial functional signatures associated with host health. These findings highlight biosynthetic and antimicrobial potential as health-associated microbial features and provide candidate microbial biomarkers and functional targets for improving resilience to post-weaning diarrhea in sustainable swine production systems.

Key Words: Swine gut microbiota, Culturomics, Biosynthetic gene clusters, Antimicrobial peptides, Post weaning diarrhea

3.2 Introduction

The gastrointestinal tract of swine harbors a dense and dynamic microbial community that plays a fundamental role in host nutrition, immune development, and disease resistance. A growing body of evidence links the gut microbiome to key production traits, including growth performance, and resilience to common diseases such as post-weaning diarrhea (PWD) (290,291). As swine production systems aim to minimize antimicrobial usage and adopt precision-fed models, there is increasing interest in harnessing microbiome-based strategies to promote health and productivity through targeted manipulation of microbial functions. Over the past decade, culture-independent approaches, including 16S rRNA amplicon and shotgun metagenomic sequencing, have provided important insights into the taxonomic composition and functional potential of the swine gut microbiome (292–296). However, much of this knowledge remains correlative, as the lack of high-quality assemblies for isolates constrain efforts to assign functions to specific microbes, validate predictions, or translate findings into actionable interventions.

Beyond taxonomy, efforts have focused on genome-resolved functional features, including biosynthetic gene clusters (BGCs), metabolic gene clusters (MGCs), antimicrobial peptides (AMPs), antimicrobial resistance genes (ARG), and carbohydrate-active enzymes (CAZymes), which play key roles in shaping microbe-microbe and microbe-host interactions and the overall resilience of the gut microbiome (297–300). However, most studies characterize these traits from metagenome-assembled genomes (MAGs) or unbinned contigs, which often suffer from inherent limitations including contamination, or incomplete binning. These challenges hinder the confident reconstruction of complete functional pathways and complicate comparative analyses across individuals or conditions. Critically, MAG-based approaches rarely enable linkage of predicted functions to cultivable strains, limiting the ability to experimentally validate or exploit these traits for translational applications. The Pig Intestinal Bacterial Collection (PiBac) (301) pioneered culturomics of the swine gut microbiota by sequencing the genomes of 110 unique bacterial species, including members of 16 novel genera. More recently, other studies have also contributed to expanding the genomic blueprint of culturable diversity of the swine gut and respiratory microbiota (302,303). However, to fully

capture the functional diversity present in the swine gut, as inferred from metagenomic surveys (304,305), expanding isolate-based genomic resources remains critical.

The goal of this study was to build on existing culture collections and expand on the strain diversity and genomic landscape of the culturable swine gut microbiota. By leveraging independent metagenomic datasets from healthy and diseased pig populations, we sought to identify potential health-associated biomarkers. Leveraging this expanded genomic resource, we (i) characterized the taxonomic breadth, biosynthetic and metabolic capacity of swine gut microbes at genome resolution, (ii) annotated key functional traits, including antimicrobial resistance genes, virulence factors, and specialized metabolic pathways, and (iii) applied machine learning to associate functional profiles with host health phenotypes. Collectively, this work underscores the potential of culture-resolved functional signatures to identify candidate taxa and traits linked to microbiome-associated health outcomes.

3.3 Methods

3.3.1 Swine Fecal and Tissue Sample Collection and Bacterial Culturing

Swine fecal and tissue samples were collected as part of larger clinical and observational studies in the CL2 Animal Isolation Facility and on a research farms at the University of Guelph (Guelph, ON, Canada; approved by the University of Guelph Animal Care Committee, AUP#4280) and the University of Manitoba (Winnipeg, MB, Canada; approved by the University of Manitoba Animal Care Committee, AC#11879), in accordance with Canadian Council on Animal Care guidelines (CCAC, 2009). Fecal samples were collected from two sows on the research farm of University of Guelph. Ileum and tissue samples were collected from their piglets in the Animal Isolation facility at Ontario Veterinary College, University of Guelph and transferred to collaborating lab at McMaster University for culturomics. At the University of Manitoba, fresh fecal samples were collected from clinically healthy animals at the Glenlea Research station, including 20 sows and eight piglets, all with no gastrointestinal symptoms and no history of antimicrobial therapy within three months of collection. Immediately after collection, samples were transferred to a sterile container and stored in sealed bags containing an anaerobic pouch (GasPak™ EZ; BD, MD, USA) and icepack. Upon arrival, samples were processed inside an anaerobic chamber (5% CO₂, 5% H₂, 90% N₂), homogenized in Brain Heart Infusion (BHI) broth, and serially diluted (10⁻¹-10⁻⁶). Dilutions were plated in duplicate (100μL) on a range of rich and selective media: BHI, fastidious anaerobe agar, gifu anaerobic medium, columbia blood agar, and phenylethyl alcohol agar to capture broad microbial diversity. Plates were incubated aerobically (one day) or anaerobically (five days) at 37°C. Individual colonies were picked, re-streaked to purity, and classified via PCR amplification, followed by sequencing of the V3 region of the 16S rRNA gene on an Illumina MiSeq platform. The resulting V3 sequences were then clustered based on 97% sequence identity using CD-HIT (306) to identify non-redundant isolate groups prior to whole genome sequencing, as previously described (307).

3.3.2 Sample Collection From Healthy and PWD Pig Cohorts

Rectal swabs were collected from 5 healthy and 10 pigs with post-weaning diarrhea (PWD) across seven commercial swine farms in Alberta and Manitoba, yielding a total of 104

samples (approved by the University of Guelph Animal Care Committee, AUP# 5194). Healthy and PWD samples were collected concurrently from the same farms using identical sampling and processing protocols to minimize batch effects. These data were used for model development, where model training and hyperparameter tuning were performed using nested cross-validation, with the outer folds providing internal validation. Model performance and generalizability across farms were further evaluated using leave-one-farm-out approach. An independent cohort of health and PWD piglets were used for external validation of the machine learning framework, comprising rectal swabs from 24 healthy and 25 PWD piglets (n = 49) collected at the University of Guelph under separate ethical approval (AUP# 5203). This dataset was not used during model training or tuning and served exclusively to evaluate model generalizability to an independent cohort. A summary of sample sources, study components, and their downstream applications is provided in **Table S5 and S6**.

3.3.3 Genome Extraction, Sequencing, Processing and Assembly

Genomic and metagenomic DNA of all isolates and fecal samples were extracted using the E.Z.N.A. Genomic DNA Isolation Kit (Omega Bio-tek, Inc.) following the manufacturer's recommendations. DNA concentrations were quantified by Qubit dsDNA HS kit (ThermoFisher Scientific, Mississauga, ON, Canada). Illumina libraries were prepared according to a miniaturized library preparation protocol as previously described (308) using the NEBNext Ultra II FS DNA Library Prep Kit (NEB, MA, USA). Libraries were subjected to dual size-selection using the ProNex® Size-Selective Purification System (Promega, WI, USA) to enrich for insert sizes of 400-600 bp. Final libraries were sequenced on an Illumina NovaSeq 6000 using a paired-end read length of 2x150 bp at Génome Québec (Québec, QC, Canada). Reads were quality-filtered, adapter-trimmed and poly-G tail removed with fastp (v0.24.1) (309), and assemblies were generated with SPAdes (v4.1.0) (310). Assembly statistics, including genome size, and GC content, were calculated for each genome using seqkit (v2.10.1). Pairwise average nucleotide identity (ANI) was computed with FastANI (v1.34) (311). Assembled genome quality was assessed using CheckM2 (v1.0.2) (312) as implemented within GTDB-Tk (v2.4.0) (313).

3.3.4 Species-level Clustering, Phylogenetic and Taxonomic Analyses

SGI genomes were combined with 110 publicly available PiBac genomes (total $n=410$) and dereplicated with dRep (v3.6.2) (314). Following the dRep two-stage framework, genomes were first grouped by Mash distance into primary clusters at $\geq 93\%$ ANI (a computational pre-filter), and each primary cluster was then refined by pairwise gANI comparison into secondary clusters at $\geq 98\%$ ANI for species level delineation. The highest-quality genome from each secondary cluster was selected as the representative, while additional conspecific strains originating from different pigs were retained to capture inter-host diversity. Genomes were taxonomically annotated and placed in a phylogenomic framework using GTDB-Tk (v2.4.0, database release 220) with the *de novo* wf pipeline (313). *Patescibacteria* was specified as the outgroup, reference genomes were excluded, and phylogenetic trees were reconstructed from concatenated bacterial marker genes using FastTree.

3.3.5 Functional Characterization

Functional potential of the SGI and PiBac genomes was assessed using multiple complementary annotation tools. antiSMASH (v7.1.0) was used to identify biosynthetic gene clusters (BGCs) (315). BGC clustering was performed using BiG-SCAPE (v2.0; cutoff = 0.3) (316), which integrates domain architecture and sequence similarity to generate a sequence similarity network. gutSMASH (v1.0) was applied to detect primary metabolic gene clusters (317). Antimicrobial resistance determinants were annotated with the Resistance Gene Identifier (RGI) (v6.0) using the CARD database v4.0 (318). Only high-confidence predictions classified as ‘strict’ or ‘perfect’ and exhibiting $\geq 90\%$ reference coverage and $\geq 90\%$ sequence identity were retained. Putative antimicrobial peptides (AMPs) were predicted with Macrel (not formally versioned), which integrates machine learning and sequence features to classify short peptides (319). Virulence factors were identified through homology-based searches against the VFDB database (320). Carbohydrate-active enzymes (CAZymes) were annotated using dbCAN3 (v5.0.6) (321).

3.3.6 Chemical Similarity Analysis

Structural similarity between MIBiG reference compounds was assessed using Tanimoto coefficients (322) in R. SMILES representations of the compounds were parsed from

PubChem and converted to extended molecular fingerprints using the *rdck* package (v3.8.1) (323). Pairwise Tanimoto similarity scores were calculated for all compound pairs, where similarity values range from 0 (no structural similarity) to 1 (identical structures).

3.3.7 Metagenomic Read Recruitment and CPM Calculation

To estimate the abundance of genome-derived functional features in healthy and PWD metagenomes, custom nucleotide databases were constructed from outputs of dbCAN3 (CAZymes) (321), antiSMASH (biosynthetic gene clusters) (315), gutSMASH (metabolic gene clusters) (317), CARD (antimicrobial resistance genes) (318), VFDB (virulence factors) (320), and Macrel (antimicrobial peptides) (319). Coding sequences from antiSMASH and gutSMASH GenBank (.gbk) outputs were converted to nucleotide FASTA. Macrel peptide predictions were converted into nucleotide sequences by aligning the peptide outputs against the corresponding genome assemblies using *tblastn* and extracting the matched coding regions in FASTA format. dbCAN3-annotated CAZyme proteins were mapped to Prodigal-predicted ORFs to retrieve their nucleotide sequences. All feature-specific FASTAs were concatenated, sequence-based deduplicated using *seqkit* (v2.11) (324) and used as reference databases for BWA (v0.7.19) (325) mapping of quality-filtered metagenomic reads. To ensure high specificity and minimize cross-mapping artifacts, particularly for short features, stringent post-mapping filtering criteria were applied. For AMPs, features were retained only if they exhibited $\geq 70\%$ breadth of coverage, mean sequencing depth $\geq 5\times$, and at least 15 covered bases. For BGCs, features were retained using criteria of $\geq 75\%$ coverage, mean depth $\geq 3\times$, and ≥ 500 covered bases. For MGCs, CAZymes, and VFs features were required to have $\geq 70\%$ coverage, mean depth $\geq 3\times$, and ≥ 100 covered bases. Reference databases were curated and deduplicated where applicable to reduce redundancy and minimize multi-mapping. Abundance estimates were derived from coverage-based metrics and normalized using count per million (CPM), defined as:

$$CPM_i = \frac{C_i \times 10^6}{N}$$

where C_i is the number of reads mapped to feature i , N is the total number of mapped reads in the sample, and 10^6 is the scaling factor.

3.3.7.1 AMP Density Calculation

AMP density was estimated per genome by predicting candidate antimicrobial peptides (cAMPs) with Macrel (319), retaining sequences with an AMP probability ≥ 0.5 . The number of unique cAMPs ($n_{cAMP,i}$) was normalized by genome size ($Length_i$) to yield density values defined as:

$$\rho_{AMP,i} = \frac{n_{cAMP,i}}{Length_i}$$

which yields cAMPs per base pair. To facilitate interpretation, densities were reported as cAMPs per megabase by scaling as $\rho_{AMP,i}^{(Mb)} = \rho_{AMP,i} \times 10^6$. When aggregated at higher taxonomic levels, both n_{cAMP} and $Length$ were summed across genomes prior to recomputing ρ_{AMP} .

3.3.7.2 ML models

Per-feature CPM values (from metagenomic read recruitment) were first aggregated by summing across all features belonging to the same functional class within each source genome, yielding one value per sample $\times$ genome $\times$ functional-class combination. To address class imbalance, model-specific class weighting strategies were applied, including class weight='balanced' for logistic and ridge regression, balanced subsample for random forest, and scale pos weight for XGBoost, in combination with stratified sampling in all cross-validation procedures. To ensure robust performance estimation and avoid overfitting, model training and evaluation were conducted using a nested cross-validation framework, consisting of an outer 5-fold stratified loop for performance estimation and an inner 3-fold loop for hyperparameter optimization. All feature preprocessing steps were performed strictly within training data in each cross-validation iteration to prevent information leakage. These steps included variance-based filtering to retain the most informative features, removal of highly correlated features based on Pearson correlation ($|r| > 0.95$) to reduce redundancy, and feature scaling using standardization. Model performance was evaluated using both nested 5 $\times$ 3-fold cross-validation and leave-one-farm-out (LOFO) validation to assess predictive robustness and cross-cohort generalizability. Feature importance was assessed using Shapley Additive explanations (SHAP), with values computed from models trained within the nested cross-validation

framework. The classification accuracy of each model was calculated using the following formula:

$$Accuracy = \frac{TP + TN}{TP + TN + FP + FN}$$

where *TP* represents true positives, *TN* true negatives, *FP* false positives, and *FN* false negatives.

3.3.7.3 Statistical analysis

All statistical analyses were performed using RStudio (version: 2025.09.0; R 4.5.0) or python (version 3.12). Differences in feature abundance between healthy and PWD groups were assessed using the Wilcoxon rank-sum test, while comparisons involving more than two groups were performed using the Kruskal-Wallis test followed by post-hoc pairwise comparisons where applicable. To control for multiple hypothesis testing across high-dimensional feature sets, *p*-values were adjusted using the Benjamini-Hochberg false discovery rate correction within each feature class, unless stated otherwise. Adjusted *p*-values < 0.05 were considered statistically significant.

3.4 Results

3.4.1 Genomic Landscape of Swine Microbiome Isolates

To expand the genomic representation of culturable members of the swine gut microbiota, we generated a collection of 1,200 gut isolates, recovered from different sections of the gastrointestinal tract content of 28 pigs. Species-level dereplication based on average nucleotide identity (ANI $\geq$ 98%) reduced these to 300 high-quality assemblies (referred to as SGI collection hereafter), while retaining conspecific strains from different hosts to capture inter-individual diversity (**Table S1**). These 300 genomes represent 88 unique species across 60 genera (**Fig. 1**). To contextualize the SGI isolates within existing culture-based isolate resources, we compared them to the 110 publicly available genomes from the PiBac collection (301). A substantial proportion of SGI genomes (49.7%; *n* = 149) lacked a close match (ANI < 98%) to the previously characterized species in the PiBac collection, with 60 showing no detectable hit (ANI < 80%) and 89 exhibiting only distant similarity (80% $\leq$ ANI < 98%). This

highlights the contribution of our collection in capturing uncultured members of the swine gut microbiota. The SGI genomes displayed a wide range of genomic features (**Fig. 1** and **Fig. S1**),

with sizes spanning 1.57 Mbp (*Helicobacter*) - 7.56 (*Streptomyces*) Mbp (median ~2.10 Mb) and GC content ranging from 27.9-73.1% (median 40.7%).

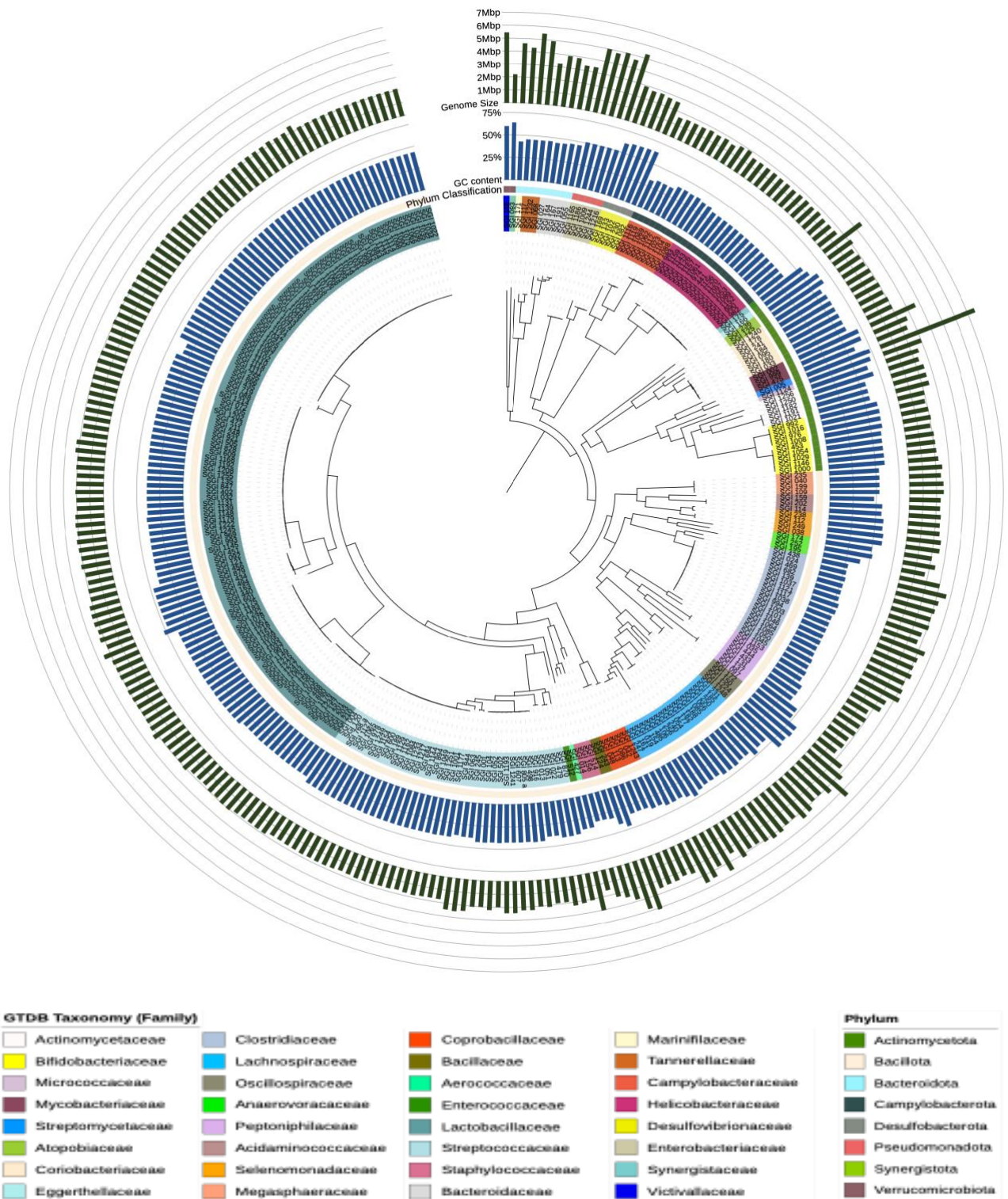


Figure 1. Genomic landscape of the SGI collection. Phylogenomic tree of 300 non-redundant bacterial isolates recovered from the swine gut (SGI). The tree was constructed de novo using GTDB marker genes and inferred with FastTree using a maximum-likelihood approach. Taxonomic assignments were performed using GTDB-Tk (v2.4.1). Concentric outer rings display genome characteristics for each isolate, including GC content (%) shown in blue and genome size (Mbp) shown in green, where bar length corresponds to the relative value for each genome. The inner taxonomic ring is color-coded by bacterial phylum, while the innermost clade colors represent family-level taxonomic classification of each isolate genome. Colors and branch annotations correspond to the taxonomic groups indicated in the accompanying legends.

Quality assessment revealed that the SGI genomes are well-resolved, with most assemblies nearing complete reconstruction (median completeness 99.4%) and containing negligible contamination (median 0.37%) (**Fig. S1A-B**). To enhance the thoroughness and impact of downstream annotation of specialized metabolic and biosynthetic pathways, we compiled our in-house SGI genome collection with 110 publicly available genomes from the PiBac collection, resulting in a unified dataset of 410 assemblies. The newly isolated genomes substantially broaden the taxonomic and functional coverage of the porcine gut microbiome. GTDB-Tk classification (313) placed the 410 genomes across thirteen phyla and twelve classes, with *Bacilli* and *Clostridia* comprising the majority, consistent with the dominant lineages of the pig gastrointestinal tract (326,327).

3.4.2 Swine Gut Isolates Harbour a Rich Repertoire of Biosynthetic Gene Clusters

The gut microbiome harbors a wide array of biosynthetic gene clusters (BGCs) that encode natural products involved in interspecies interactions (328,329), colonization resistance (330–332), and modulation of host immunity and physiology (333–335). To assess the biosynthetic potential of swine gut isolates, we first predicted BGCs across the unified collection using antiSMASH (315). In total, 1,246 BGCs were identified, spanning diverse secondary metabolite classes (**Fig. 2A**). Most BGCs were class-specific, with limited overlap between major classes, while hybrid or multi-class BGCs were comparatively rare. Ribosomally synthesized and post-translationally modified peptides (RiPPs) were the most abundant category (503 BGCs; 40.4%), reflecting the dominance of peptide-based natural products among gut-associated bacteria. This was followed by terpenes (310 BGCs; 24.9%),

polyketide synthases (PKSs) (196 BGCs; 15.7%), and non-ribosomal peptide synthetases (NRPSs) (94 BGCs; 7.5%). Additionally, 49 hybrid BGCs (3.9%) were identified, and the remaining 94 BGCs (7.5%) were classified as belonging to other or uncharacterized classes.

At the taxonomic level, BGC distributions were markedly non-uniform across bacterial orders (**Fig. 2B**). The Z-score normalization highlighted distinct order-specific biosynthetic signatures, revealing taxon-dependent enrichment of specific secondary metabolites classes. Orders such as *Lachnospirales*, *Clostridiales*, *Tissierellales*, *Peptostreptococcales*, *Erysipelotrichales*, *Victivallales*, *Aneurinibacillales*, *Eubacteriales*, *Selenomonadales*, *Oscillspirales*, *Veillonellales* and *Acidaminococcales* exhibited strong enrichment for RiPPs, whereas *Burkholderiales* and *Mycobacteriales* were enriched in PKS and NRPS, respectively, consistent with their well-known role as prolific producers of complex polyketides and non-ribosomal peptides (336).

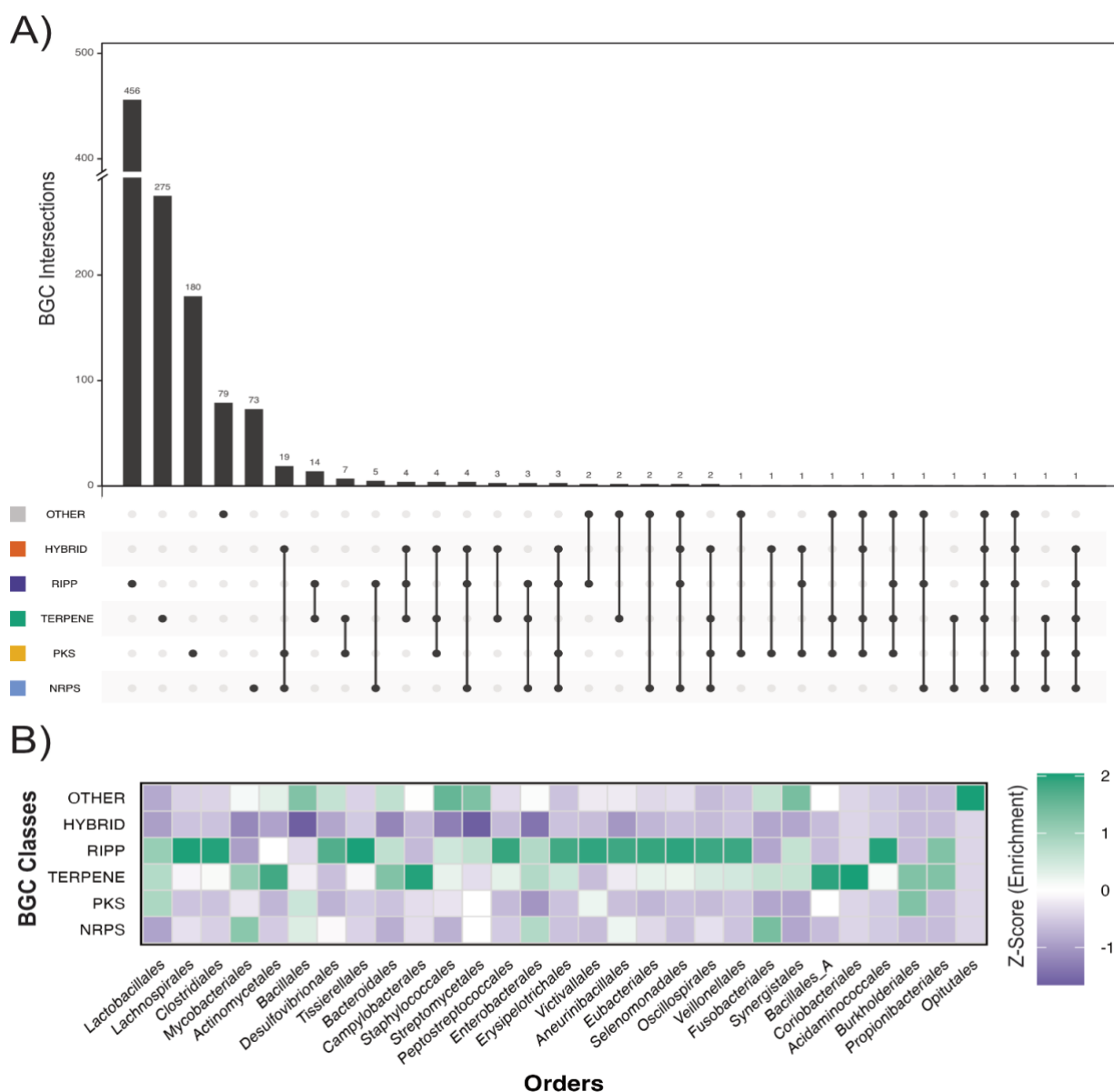


Figure 2. BGC diversity in swine gut isolates. A) Intersections and distribution of BGC classes across the swine gut genome collection. Horizontal bars indicate the total number of BGCs assigned to each class, colored by class type: NRPS (blue), PKS (yellow), Terpenes (green), RIPP (purple), HYBRID (orange), and OTHER (gray). Vertical bars represent the size of each unique class combination, with connected dots below indicating the contributing BGC classes. **B)** Order-level enrichment of BGC categories. Colors indicate enrichment relative to the mean BGC profile of each order (Z-score).

3.4.3 Sequence Similarity Network Reveals Previously Uncharacterized Biosynthetic Gene Families and Disease-Associated Depletion in BGC Abundance

To explore the structural and functional diversity of the predicted BGCs within the swine gut microbiome, we constructed a sequence similarity network based on domain architecture using BiG-SCAPE (similarity cutoff = 0.3) (316). We reasoned that a permissive threshold would better capture both closely related clusters and more divergent homologs that may encode similar chemistry. The resulting sequence similarity network resolved 191 gene cluster families (GCFs), including 25 families with matches to known MIBiG reference clusters (**Table S2**) and 166 GCFs with no corresponding MIBiG hits (**Fig. 3A**). These MIBiG-unassigned GCFs represent previously uncharacterized and potentially novel biosynthetic families, highlighting the unexplored biosynthetic potential of swine gut microbes. These uncharacterized GCFs spanned diverse BGC classes, including predicted NRPS, RiPP, terpene, and hybrid classes. Notably, many of these uncharacterized GCFs were contributed exclusively by the SGI isolates (**Table S2**), demonstrating that expanding culture-based efforts can substantially reveal hidden biosynthetic diversity. Within the GCFs, distinct but biosynthetically related natural products were clustered, indicating that SSN connectivity reflects shared biosynthetic architecture. For example, a GCF composed exclusively of known glycopeptide BGCs (denoted as GCF X in **Fig. 3A**), encompassed multiple distinct antimicrobials, including balhimycin, chloroeremomycin, ristocetin and its variants, vancomycin, decaplanin, actinoidin B, avoparcin, and keratinimicin variants. Pairwise chemical similarity analysis of representative glycopeptides from this MIBiG-anchored GCF revealed high structural conservation (Tanimoto coefficients: >0.80) reflecting their conserved core scaffold, while resolving into distinct subgroups through variable modifications (**Fig. S2**).

Several large GCFs exhibited extensive connectivity, while many smaller families contained only a handful of putatively novel nodes, suggesting widespread biosynthetic capacities. To evaluate the functional relevance of uncharacterized BGCs in the swine gut microbiome, we focused on GCFs that lacked homology to any known MIBiG reference clusters. Because these non-MIBiG GCFs comprise the majority of BGC diversity in our dataset and may encode previously uncharacterized metabolites, we prioritized them for ecological profiling. To contextualize these uncharacterized BGCs within host-associated communities,

we performed metagenomic read recruitment, mapping the BGCs against metagenomes from healthy and post-weaning diarrhea (PWD)-affected piglets to assess their *in-situ* prevalence. For each GCF, we quantified (i) BGC richness, calculated as the median number of distinct BGCs detected per sample within each group, and (ii) its relative abundance across samples based on normalized read count. A majority of the analyzed GCFs exhibited a consistent and statistically significant (Wilcoxon rank-sum test, with BH correction) reduction in both richness and abundance in PWD samples relative to healthy controls (**Fig. 3B**), underscoring their potential relevance to microbiome function and gut health. These clusters likely encode metabolites involved in niche colonization and microbial interactions, and their depletion in PWD samples may be indicative of a reduction in biosynthetically active taxa and community metabolic capacity during dysbiosis. Together, these findings highlight the extensive and previously uncharacterized biosynthetic repertoire of the swine gut microbiome and show that PWD is associated with marked changes in the diversity and abundance of these biosynthetic capacities.

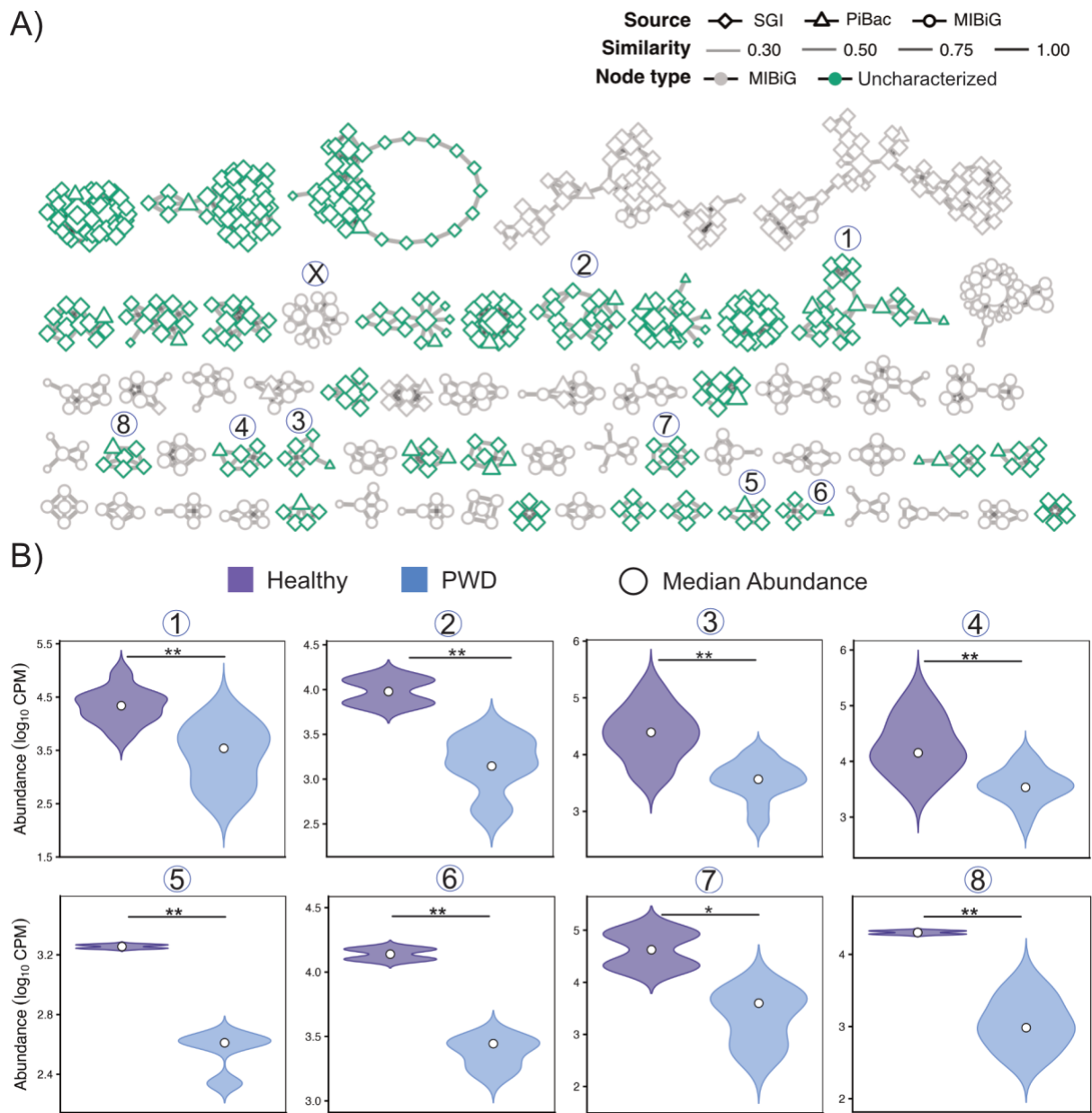


Figure 3. GCF network structure and disease-associated shifts in abundance. **A)** Sequence similarity network of BGCs grouped into GCFs based on domain architecture similarity. Nodes represent individual BGCs from the MIBiG (circles), PiBac (triangles), or SGI (diamonds) collections. Edges reflect pairwise similarity scores between clusters (edge width scales with similarity). **B)** Distribution of gene cluster families (GCFs) with statistically significant differences between healthy and post-weaning diarrhea (PWD) piglets in metagenomic datasets. Statistical significance was determined by Wilcoxon rank-sum test with p-values adjusted using the Benjamini-Hochberg (BH) method. Significance levels are denoted as ** $p < 0.01$, * $p < 0.05$.

3.4.4 Antimicrobial Peptide Profiling and Functional Prediction

To expand the assessment of the antimicrobial potential encoded within the swine gut microbiome, we systematically identified and characterized antimicrobial peptides (AMPs) from our compiled genomic collection of swine gut isolates. Because physicochemical properties strongly influence AMP activity, specificity, and mechanism of action, we profiled key traits that govern AMP-microbe interactions, including net charge (small and basic residues) that promotes membrane binding, hydrophobicity (grand average of hydropathy; GRAVY score) that facilitates penetration through lipid bilayer, and variation in structure and stability (peptide length, aliphatic index, instability index) that shapes antimicrobial potency. We observed that the identified AMPs occupy a broad physicochemical landscape, characterized by extensive variation in charge, hydrophobicity, and structural features (**Fig. S3A**), reflecting canonical AMP traits that promote membrane disruption and antimicrobial activity.

Genome-level analysis revealed strong taxonomic structuring of AMP biosynthetic capacity. AMP density varied significantly across bacterial genera in the swine gut isolate collection (Kruskal-Wallis test with Benjamini-Hochberg correction, $p < 0.001$). Among genera with at least three representative genomes, *Prevotella*, *Enterococcus*, and *Sarcina* exhibited notably high AMP density per genome (median; **Fig. 4A**). Notably, *Prevotella* exhibited significantly higher AMP density than other genera (pairwise Wilcoxon tests, Benjamini-Hochberg adjusted $p < 0.05$; **Fig. 4A**). These genera may represent ecological hotspots for endogenous AMP production and microbe-microbe competitive dynamics within the gut environment. Most AMPs identified across swine gut isolates were predicted to be highly bioactive with low hemolytic potential (**Fig. S3B**), suggesting that the swine gut microbiome harbors peptides optimized for antimicrobial efficacy with reduced host cytotoxicity.

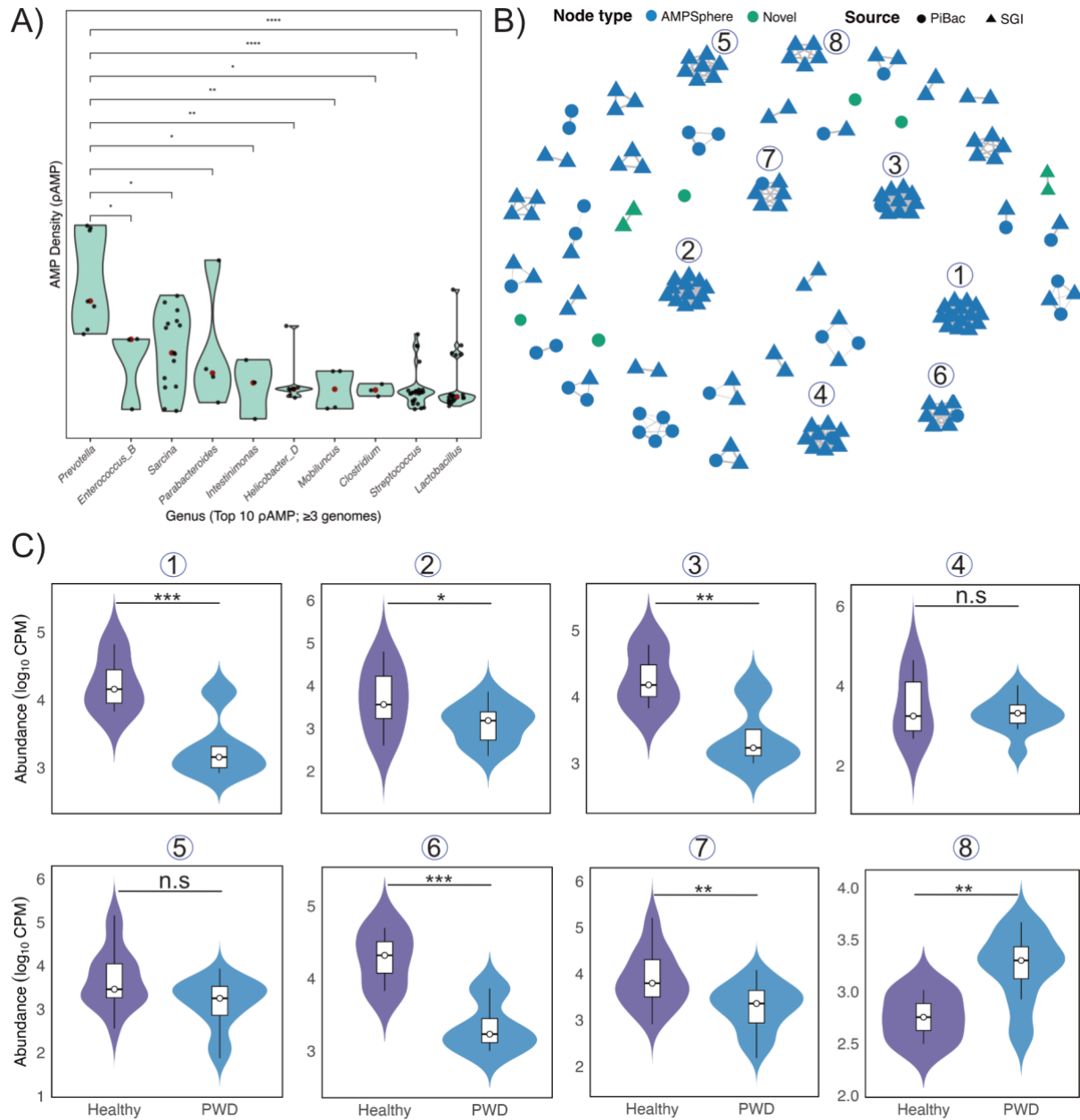


Figure 4. Abundance and distribution of AMPs across swine gut isolates. **A)** AMP density (number of predicted AMPs per genome) across the top 10 most AMP-rich bacterial genera (≥ 3 genomes per genus). Statistical significance determined by Kruskal-Wallis test with pairwise Wilcoxon tests (Benjamini-Hochberg correction; ****p < 0.0001, *** p < 0.001, ** p < 0.01, * p < 0.05). **B)** Sequence similarity network of AMPs predicted from SGI and PiBac genomes, constructed using AMPSphere reference similarity. Each node represents a unique AMP peptide, with node size reflecting percent identity (% ID) to AMPSphere hits. **C)** Relative abundance of putatively novel AMP gene cluster families (≥ 5 members per GCF) across healthy and PWD metagenomes. Violin plots show the distribution of normalized abundance

per genome, with embedded boxplots indicating median and interquartile range. Statistical significance between groups was assessed via Wilcoxon rank-sum test, with Benjamini-Hochberg (BH) correction. Significant levels are denoted as *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, n.s (not significant).

To examine AMP functional diversity and its relevance to host health status, we constructed a sequence similarity network (SSN) of predicted AMPs using AMPSphere as reference database and performed abundance profiling across the isolate genomes. The resulting SSN revealed well-defined AMP gene cluster families, encompassing both known and putatively novel AMPs (**Fig. 4B**). The presence of these putatively novel AMP clusters highlights the importance of genome-resolved approaches in expanding the known antimicrobial peptide space, as low-complexity small open-reading frames encoding AMPs may confound binning heuristics (337,338) and short reads may not span entire coding regions. We next assessed the genome-level abundance and disease group-specific enrichment of these putatively novel AMP families. Focusing on families with ≥ 5 distinct AMPs in each, we compared the abundance of these AMPs across healthy and PWD-associated metagenomes. Majority of the AMP clusters were significantly enriched (Wilcoxon rank sum test, with Benjamini-Hochberg correction) in the metagenome of healthy pigs (**Fig. 4C**). These findings suggest that AMP potential is selectively depleted in disease-associated perturbed microbiome states, mirroring the pattern observed for BGCs. Altogether, these results highlight the swine gut microbiome as a rich repertoire of diverse AMPs and reveal their loss as a hallmark of PWD-associated disruption of the microbial community.

3.4.5 Functional Stratification of Primary Metabolism and CAZyme Profiles in Swine Gut Isolates

To systematically investigate the specialized metabolic potential encoded within the swine gut isolate genomes, the gutSMASH genome mining tool was used to identify metabolic gene clusters (MGCs) associated with energy metabolism, amino acid catabolism, SCFA biosynthesis, and other gut health-relevant metabolic routes (317). This approach predicted 2,266 MGCs corresponding to 65 distinct primary metabolic pathways. To summarize functional capacity, we aggregated pathway presence data at higher-taxonomic level (Order)

and computed the number of unique genera harboring each pathway (**Fig. 5A**). The resulting order-by-pathway heatmap revealed a spectrum of taxonomic specialization and metabolic redundancy across clades. Members of the *Enterobacterales*, *Bacteroidales*, and *Lachnospirales* encoded broad and diverse metabolic repertoires, including overlapping sets of SCFA and energy-linked pathways. For instance, some *Lachnospirales* lineages carried both butyrate and propionate synthesis pathways, while others specialized in aromatic degradation. In contrast, several orders such as *Opitutales* and *Spirochaetales* displayed more constrained profiles, typically encoding only a few SCFA-related pathways. Notably, even within the same order, pathway presence varied substantially at the genus level (**Table S3**), suggesting strong intra-lineage diversification of core metabolic strategies.

To complement these insights into primary metabolism, we next investigated the carbohydrate-active enzyme (CAZyme) repertoire across the swine isolate genomes using dbCAN3 (321), which annotates enzymes involved in the breakdown, modification, and synthesis of complex carbohydrates. Given the central role of glycan utilization in shaping gut microbial ecology, we assessed both the richness and density of CAZymes across lineages to uncover patterns of enzymatic specialization. Most bacterial families were dominated by glycoside hydrolases (GH) and glycosyltransferases (GT) (**Fig. 5B**), consistent with their central roles in carbohydrate metabolism (339,340) and their prevalence in the CAZy database (341). Indeed, the unified swine gut isolate collection encompasses a substantial portion of known GH and GT diversity (**Table S4**), spanning 128 GH families and 54 GT families, corresponding to a major fraction of the GH (66%) and GT (39%) families catalogued in the CAZy database (341). Taxonomic families such as *Mycobacteriaceae*, *Megasphaeraceae*, *Acidaminococcaceae*, *Bacillaceae*, *Oscillospiraceae* and *Peptoniphilaceae* displayed a more diverse CAZyme composition, including auxiliary activities (AA), carbohydrate-binding modules (CBM), and carbohydrate esterases (CE), indicating complex glycan-processing capabilities.

To account for genome size variation, we next calculated CAZyme gene density as the number of CAZyme genes per 10 kb within the genome (**Fig. 5C**). This class-specific analysis revealed that GHs and GTs were consistently the most abundant across most families, underscoring their central roles in polysaccharide degradation and glycan remodeling.

Conversely, families such as *Oscillospiraceae*, *Anaerovoracaceae*, and *Actinomycetaceae* displayed low overall densities across all CAZy classes, suggesting more limited capacities for complex carbohydrate utilization. Interestingly, certain families showed selective enrichment for specific classes: for instance, *Bacillaceae*, and *Bacteroidaceae* had relatively elevated CE and PL densities, while *Clostridiaceae* and *Bifidobacteriaceae* exhibited high CBM representation.

Genus-level CAZyme profiles assessment uncovered striking disparities in the breadth and density of glycan-active functions across taxa. After normalizing total CAZyme counts by the number of genomes represented within each genus, *Victivallis*, *Phocaeicola*, *Eisenbergiella*, *Bacteroides* and *Parabacteroides* exhibited the highest CAZyme abundance, consistent with their broad glycan-degrading capacity observed in the human gut (342). CAZyme density further reinforced these patterns, with *Victivallis* displaying the greatest enzymatic density, followed by *Phocaeicola*, *Bacteroides*, *Ruthenibacterium* and *Parabacteroides* indicating compact genomes enriched for carbohydrate-active functions. These results implicate a small subset of genera as major contributors to glycan-processing potential within the swine gut microbiome. Together, these analyses reveal a highly functionally stratified swine gut microbiota, in which broad metabolic versatility and lineage-specific glycan-degradation strategies coexist to shape community-level nutrient processing.

and PL (polysaccharide lyases). C) Dot plot showing CAZyme gene density per class across families, calculated as the mean number of CAZyme genes per 10 kb.

3.4.6 Antimicrobial Resistance and Virulence Factor Profiles Across the Swine Gut Isolates

To assess the prevalence of antimicrobial resistance genes (ARGs) across different lineages of the swine gut bacteria, we annotated resistance genes against the Comprehensive Antibiotic Resistance Database (CARD) (318). Genome-normalized ARG content exhibited pronounced heterogeneity, with ARG densities ranging from 0.17 to 10.8 ARGs per Mbp. Nonetheless, the majority of genomes fell within a constrained interquartile interval of 0.48-1.14 ARGs per Mbp, reflecting a predominant low-to-moderate ARG load among the cultured isolates (**Fig. 6A**). Most of the resistance hits were associated with tetracyclines, lincosamides, macrolides, aminoglycosides and fluoroquinolones (**Fig. 6B**). ARG richness varied widely across bacterial families, with most families exhibiting low to moderate diversity. In contrast, Enterobacteriaceae possessed a markedly expanded and heterogeneous ARG repertoire (on average ~ 43 ARGs). Resistance mechanism profiles also differed substantially across families (**Fig. 6C**).

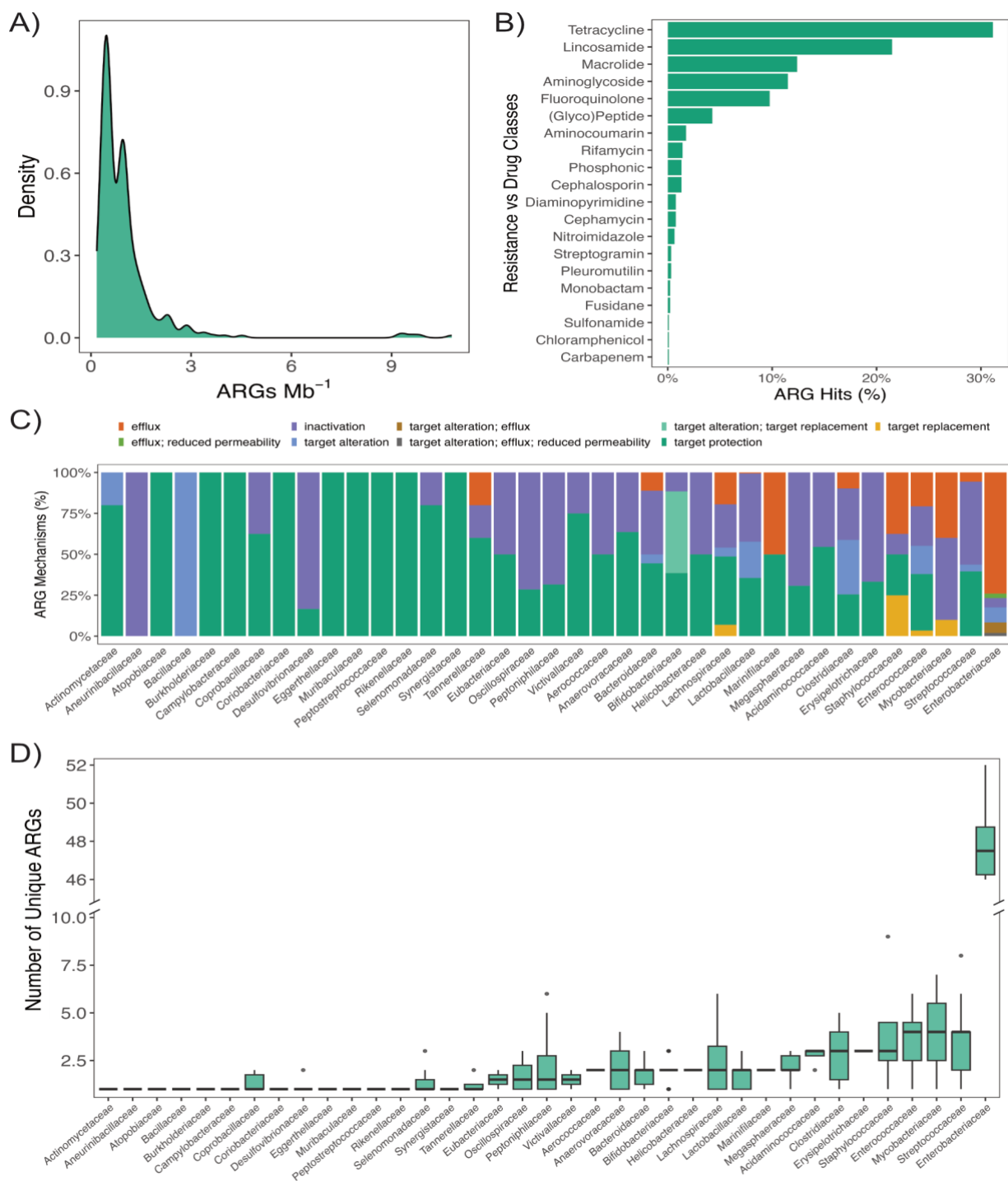


Figure 6. Functional landscape of ARGs across the swine isolate genomes. A) Distribution of ARG density across the genome collection, measured as the number of ARGs per megabase (per Mb). **B)** Relative abundance of predicted resistance genes across major drug classes based on CARD annotations. **C)** Proportion of resistance mechanisms across bacterial families,

categorized by CARD's ontological classifications. **D)** Distribution of ARG richness (number of distinct ARGs detected per genome) across all GTDB-assigned bacterial families represented in the swine gut isolates.

In several bacterial families, antimicrobial resistance was primarily driven by target protection, target inactivation and efflux-related mechanisms. Several families, including *Atopobiaceae*, *Burkholderiaceae*, *Campylobacteraceae*, *Coriobacteriaceae*, *Peptostreptococcaceae* and *Muribaculaceae* displayed highly uniform profiles, with nearly all detected ARGs linked to target protection. In contrast, families such as *Enterobacteriaceae*, *Enterococcaceae*, *Mycobacteriaceae* and *Staphylococcaceae* exhibited more heterogeneous resistance profile, with substantial contributions from efflux-mediated mechanisms, target inactivation, target alteration and multi-mechanism combinations. Together, these patterns suggest that while most commensal lineages rely on a narrow set of resistance strategies, a subset of taxa maintain broad and mechanistically diverse ARG repertoires. In parallel, putative virulence-associated features were profiled using VFDB (320). Among VF-encoding genomes, the highest burden was observed in *Enterococcus* and *Escherichia* species, which also exhibited the highest VF prevalence across the genomes (**Fig. S4**). Several *Enterococcus faecalis* and *Escherichia coli* genomes harbored multiple virulence markers related to adhesion, immune evasion, and host cell modulation. In contrast, genera such as *Enterobacter* and *Intestinimonas* had minimal VF signatures.

3.4.7 Genome-resolved Functional Signatures Enable Classification of Host Health Phenotype

To evaluate whether the genome-encoded functional landscape of swine gut isolates reflects host health status, we first performed an unsupervised projection of normalized genome functional feature (BGC, AMP, MGC, CAZyme, ARGs and VF) abundance profiles using t-distributed stochastic neighbor embedding (t-SNE) (343). This dimensionality reduction approach, followed by k-means clustering revealed trend toward distinct functional feature profiles in the healthy versus PWD hosts (**Fig. S5A**), suggesting the presence of host-specific functional signatures encoded within the microbial genomes.

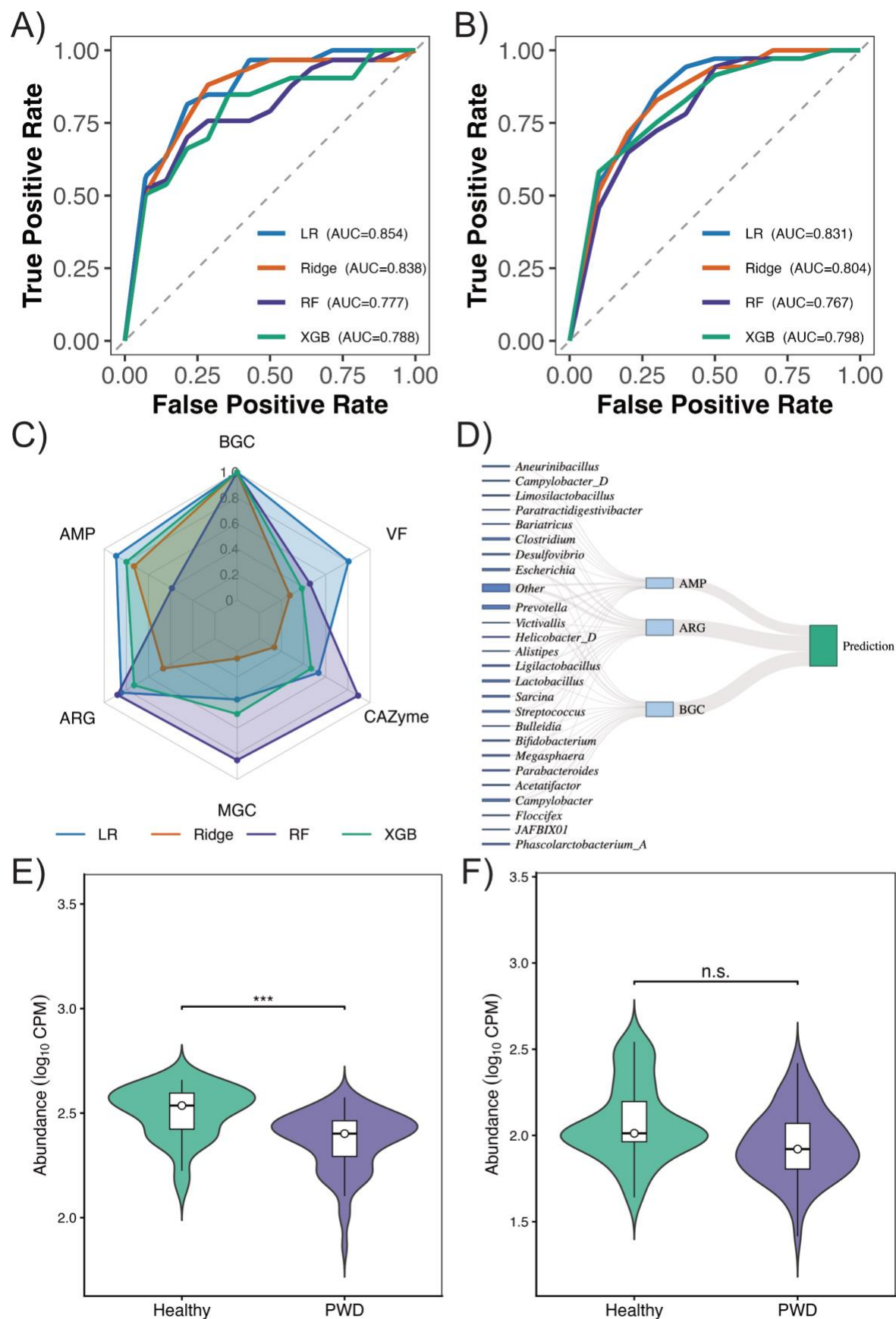


Figure 7. Machine learning distinguishes PWD and healthy samples using genome feature abundance. A-B) Receiver operating characteristic (ROC) curves showing model performance (AUROC) for logistic regression (LR), random forest (RF), XGBoost (XGB), and ridge classifiers (Ridge) in predicting sample health status. Models were evaluated using nested cross-validation (A) and leave-one-study-out (LOSO) validation (B). **C)** Spider chart showing the relative SHAP importance of feature classes (AMP, ARG, BGC, CAZyme, VF, gutSMASH) in distinguishing between health groups. Polygons represent four machine learning models: LR (blue), RF (purple), XGB (green), and Ridge (Orange). For comparability, feature importances were max-scaled within each model (largest feature set to 1.0). **D)** Sankey diagram mapping the top-contributing functional features (AMPs, BGCs, ARGs) to their corresponding bacterial genus and classification outcome based on the consensus of the XGBoost (XGB), Logistic Regression (LR), and Ridge regression models. Ribbon widths represent the averaged weighted contribution of each genus–feature association across the three models. The top 25 contributing genera are shown, with the remaining taxa grouped as “Other”. **E-F)** Distribution of the abundance of predicted bioactive features per sample, including BGCs (E) and AMPs (F), across healthy and PWD groups. Statistical significance between healthy and PWD groups was assessed using the Wilcoxon rank-sum test, with Benjamini-Hochberg (BH) correction. Significance levels are indicated as *** $p < 0.001$, and *n.s.* (not significant).

To further assess the predictive potential of these functional features, we trained four supervised machine learning classifiers, including logistic regression (LR), random forest (RF), ridge regression (Ridge) and extreme gradient boosting (XGBoost), using genome-level aggregated abundance profiles derived from six functional databases, including antimicrobial peptides (AMPs), biosynthetic gene clusters (BGCs), carbohydrate-active enzymes (CAZymes), virulence factors (VFs), antimicrobial resistance genes (ARGs), and gut-specialized metabolic pathways (gutSMASH). We aggregated features at the genome-functional class level to reduce the high dimensionality, sparsity, and multicollinearity arising from co-occurring, functionally related features within genomes (344), while retaining biologically interpretable organism-level signals for downstream machine learning analyses. We reasoned that feature-specific nucleotide mapping would better preserve biologically meaningful sequence- and genome-level functional variation than orthologous group- or protein-family-based approaches, which group homologous sequences into broad functional bins and can obscure genome-level context. Such aggregation can mask functionally distinct variants, particularly for highly diverse or rapidly evolving features such as CAZymes, where members within the same family or subfamily may differ substantially in substrate specificity ,

catalytic activity and biological function (345,346). We evaluated the model performance using both nested 5×3-fold cross-validation and leave-one-farm-out (LOFO) validation to assess predictive robustness and cross-cohort generalizability. Under nested cross-validation, linear models consistently demonstrated superior discriminative performance relative to tree-based ensemble approaches (**Fig. 7A**). LR achieved the highest mean AUROC (0.854), followed closely by ridge regression (mean AUROC = 0.838). In contrast, XGBoost and RF showed comparatively lower predictive performance, with mean AUROCs of 0.788 and 0.777, respectively (**Fig. 7A**). To further evaluate cross-cohort generalizability and minimize potential study-specific bias, models were additionally assessed using a leave-one-farm-out (LOFO) cross-validation strategy was implemented, in which samples from one farm were iteratively excluded from training and used for evaluation, while models were trained on the samples from the remaining farms. As expected, overall predictive performance declined modestly under this more stringent evaluation strategy; however, the relative ranking of classifiers remained consistent. LR yielded the strongest performance (mean AUROC = 0.831), followed by ridge regression (mean AUROC = 0.781), XGBoost (mean AUROC = 0.735), and RF (mean AUROC = 0.698) (**Fig. 7B**). Similar to the nested cross-validation, tree-based ensemble models (XGBoost and RF) demonstrated comparatively weaker discriminative performance than LR and RR. Collectively, these findings suggest that feature-level functional abundance profiles contain robust predictive signals associated with swine health status.

To interpret model predictions and identify the genome-encoded functional signatures associated with host health status, we computed SHAP (SHapley Additive exPlanations) values (347) from the nested cross-validation framework and ranked features by their contribution to the prediction (**Fig. 7B**). Notably, the best-performing models, LR and RR, consistently assigned the highest importance to biosynthetic gene clusters (BGCs) and antimicrobial peptides (AMPs), suggesting that antimicrobial and secondary metabolite-associated functions represent major discriminative signals distinguishing healthy and PWD microbiome profiles. ARGs also ranked among the top contributing feature classes in both models. In contrast, lower-performing models showed more variable feature importance patterns; RF assigned relatively greater importance to CAZymes and MGCs, whereas XGBoost retained strong contributions from BGCs together with AMPs and ARGs. The concordance between the strongest-

performing linear models in prioritizing BGCs, AMPs, and ARGs supports the biological relevance and robustness of these genome-encoded functional signatures in microbiome-based health classification.

To contextualize the taxonomic origin of the health-associated functional signatures, we mapped the top-contributing genome features (BGCs, AMPs, and ARGs) identified from the nested cross-validation SHAP analyses (**Fig. 7C**) to their originating bacterial genera and linked them to the health-associated group (**Fig. 7D**). The results show that genera such as *Prevotella*, *Aneurinibacillus*, *Paratractidigestivibacter*, and *Limosilactobacillus* were major contributors of health-enriched AMPs, while *Campylobacter*, *Lactobacillus*, *Phascolarctobacterium*, *Prevotella*, and an unclassified member (JAFBIX01) of the Lachnospiraceae family contributed prominently to BGC-associated genome features. This underscores their potential role as reservoirs of beneficial antimicrobial and biosynthetic capabilities. ARG features were more diffusely distributed across genera, with *Escherichia* and *Clostridium* contributing most strongly to the predicted signal.

Prompted by these findings, we next examined whether predicted bioactive functional elements differed between the healthy and PWD hosts. We leveraged previously developed machine learning algorithms to predict the bioactivity of the identified BGCs (348) and AMPs (319). Metagenomic abundance profiles were then subset to include only predicted bioactive features, and cumulative CPM abundances were compared between healthy and PWD samples. While the number of predicted bioactive BGCs and AMPs are not significantly different between the health groups (**Fig. S5B-C**), we observed a significantly higher abundance (CPM) of predicted bioactive BGCs in healthy hosts compared to PWD hosts (**Fig. 7E**; Wilcoxon rank-sum test with BH correction, $p < 0.0001$). Predicted bioactive AMPs also showed higher abundance in healthy hosts, although the difference was not statistically significant (**Fig. 7F**). Together, these findings indicate that host health status is associated not only with taxonomic and functional shifts, but also with enrichment of genome-encoded antimicrobial and biosynthetic potential. These findings establish a foundation for potential biomarker discovery and genome-based diagnostics and highlight specific bacterial families and functional traits as promising targets for microbiome-based interventions.

3.4.8 Cross-Cohort Evaluation Supports Classification Performance of Functional Feature-Based Models

To evaluate the generalizability of the machine learning models across independent cohorts, we applied the trained classifiers to an independent swine metagenomic dataset consisting of healthy (n = 24) and PWD (n = 25) samples. Among the evaluated models, Ridge regression achieved the highest predictive performance, with an AUROC of 0.774 and an overall classification accuracy of 0.78 (**Fig. 8A-B**). Logistic regression also showed strong performance (AUROC = 0.748; accuracy = 0.73), whereas XGBoost achieved a lower AUROC (0.691) but retained comparable classification accuracy (accuracy = 0.76). Evaluation of the confusion matrices revealed distinct error profiles across the models (**Fig. 8C**). Ridge regression provided the most balanced classification performance, correctly identifying 19 healthy and 19 PWD samples, with relatively few false predictions. Logistic regression showed higher sensitivity toward PWD classification, correctly identifying 20 PWD samples but misclassifying a larger proportion of healthy samples. In contrast, XGBoost achieved the highest correct classification of healthy samples (22/24) but showed reduced sensitivity for detecting PWD samples, correctly classifying 15 PWD samples. Overall, these results show that genome feature abundance-based ML models retain predictive capacity across independent datasets, supporting the robustness and cross-cohort generalizability of the identified microbiome-associated functional signatures.

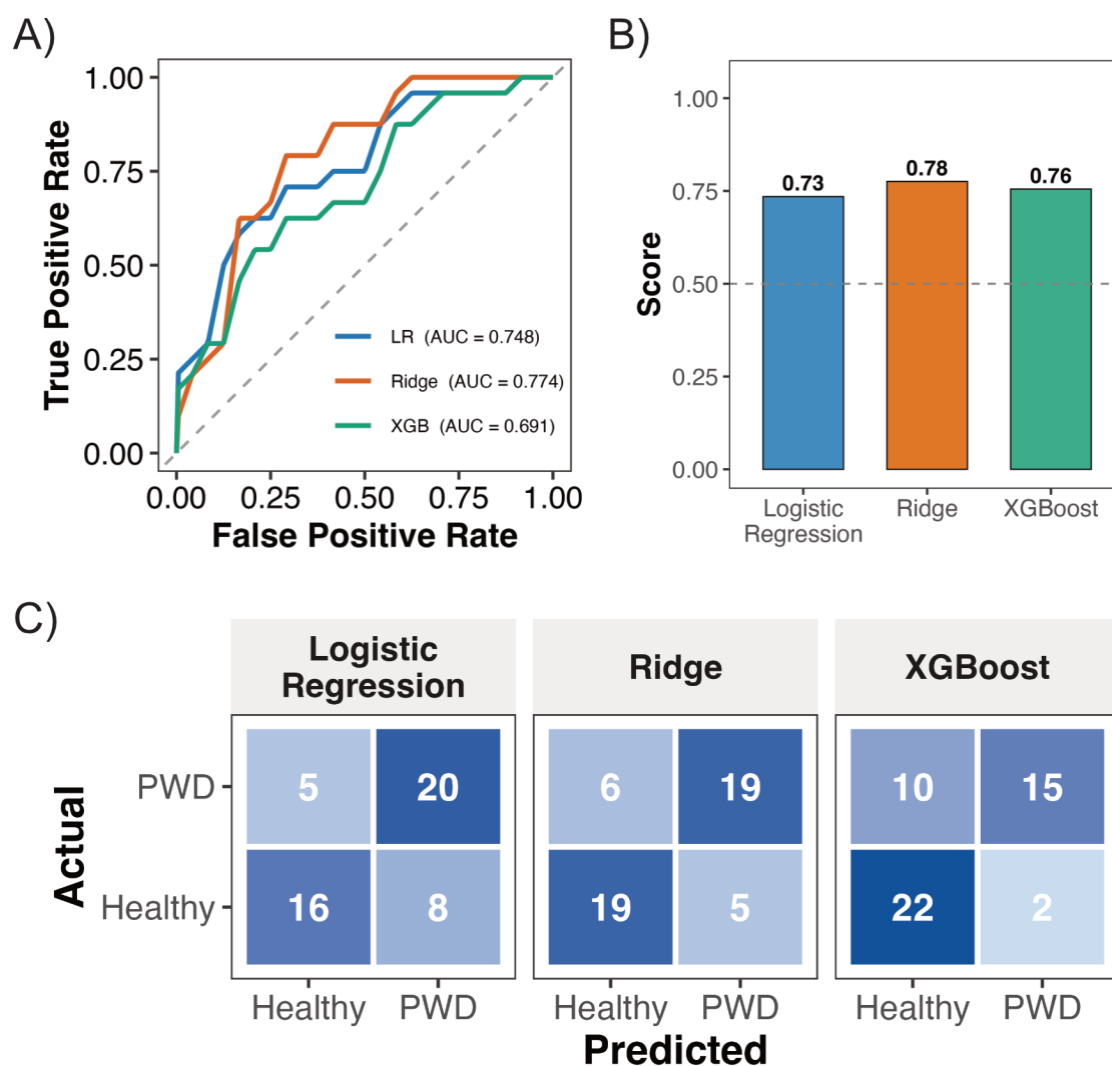


Figure 8. Cross-cohort validation of machine learning models distinguishing healthy from post-weaning diarrhea (PWD) samples on an independent swine metagenomic dataset. A) Receiver operating characteristic (ROC) curves for the top-performing classifiers (RR, LR, and XGB) trained on genome feature abundance profiles. **B)** Overall classification accuracy of each model on the independent dataset. **C)** Confusion matrix summarizing the classification outcomes of the best-performing models.

3.5 Discussion

In this study, we expanded the culture-based genomic resources for the swine gut microbiome by adding 300 newly generated, unique bacterial isolate genome assemblies to the

existing PiBac (301), yielding a unified catalog of 410 high-quality genomes. This expanded reference set substantially broadens the known taxonomic and functional diversity of the porcine intestinal microbiome, spanning 49 families across 13 phyla. This unified catalog includes previously uncultured or functionally unexplored lineages and provides a foundation for analyzing biosynthetic capacity, antimicrobial functions, and disease-associated functional profiles.

Beyond taxonomic expansion, this genomic catalog revealed extensive functional potential encoded within swine gut bacteria, including diverse BGCs, MGCs, CAZymes, AMPs, ARGs, and virulence factors. RiPPs and terpenes were the most prevalent BGC classes (**Fig. 2**), consistent with the dominance of peptide-based natural products in host-associated microbes (349). Our analyses identified numerous previously uncharacterized BGCs with no detectable homology to MIBiG entries (**Fig. 3A**), including several unique to the SGI collection. This suggests that the swine gut environment may promote the evolution of distinct biosynthetic pathways that contribute to regulating intestinal physiology and metabolic homeostasis in pigs. BGC-encoded specialized metabolites are known to affect niche colonization, mediate microbial competition, or modulate host interactions (350). The marked depletion of several BGC families in PWD-associated hosts (**Fig. 3B**) suggests a loss of biosynthetically active taxa and a consequent reduction in the functional metabolic capacity of the microbiome community during dysbiosis. These observations suggest that specific biosynthetic functions may contribute to microbiome stability and resilience, underscoring the importance of natural product potential as a dimension of microbiome function.

Antimicrobial peptides, particularly those encoded by *Prevotella*, *Aneurinibacillus*, *Paratractidigestivibacter*, and *Limosilactobacillus*, were enriched in healthy pigs and showed high predicted bioactivity with low hemolytic potential (**Fig. S3**). Their marked depletion in PWD-associated metagenomes (**Fig. 4B**) reflects a broader loss of biosynthetically active taxa during dysbiosis. Although these functional shifts are likely consequences of PWD rather than primary drivers, they nonetheless indicate a depletion of key microbiome-derived mechanisms, such as colonization resistance and microbial competition, that normally support gut barrier function and suppress opportunistic pathogens. This reduced functional capacity underscores

how disease-associated dysbiosis can deplete metabolic and genetic capabilities of the microbiome that are essential for maintaining intestinal homeostasis (351–353).

The broad yet lineage-specific distribution of metabolic gene clusters reflects ecological specialization in primary metabolism and SCFA biosynthesis, consistent with the observation in human gut microbiota (317). CAZyme profiling highlighted divergent glycan utilization strategies, with some families encoding extensive repertoires of GHs, GTs, and CBMs, while others possessed comparatively narrow sets of CAZymes (**Fig. 5**). These extensive and diverse CAZyme repertoires underscore that the expansion of carbohydrate degrading capacity not only confers ecological and competitive advantages but also constitutes a considerable allocation of genomic resources within microbial lineages (354,355). The combination of gutSMASH and CAZyme analyses reveals both broad-spectrum degradative potential and clade-specific metabolic signatures, suggesting ecological partitioning of glycan utilization and primary metabolic functions. These patterns highlight how functionally diverse lineages may play central roles in nutrient processing, while other families exhibit more specialized or streamlined metabolic strategies. Together, these patterns indicate that metabolic flexibility and antimicrobial capacity are key dimensions of microbial adaptation in the swine gut and may underpin functional shifts associated with health or disease states.

The ARG composition of our isolate collection is congruent with resistome patterns reported in previous surveys of the swine gut microbiome with tetracycline, lincosamide, macrolide and aminoglycoside resistance to be among the most prevalent ARGs in the swine gut microbiome (356,357). These recurrent signatures across studies likely reflect long-standing antimicrobial selection pressures in swine production, where tetracyclines have been extensively deployed over multiple decades (358), and lincosamides and macrolides remain routinely administered for common infections in livestock herds (359). While most bacterial families exhibited limited ARG richness, *Enterobacteriaceae* possessed a substantially expanded and mechanistically heterogeneous ARG repertoire, mirroring prior observations (360,361).

Virulence factor annotation posed interpretive challenges due to the limited scope of the virulence factor database (VFDB), which is biased toward clinically relevant and well-

characterized genera (362). As a result, virulence genes were largely concentrated in a few taxa. Nevertheless, VFDB includes key swine pathogens such as ETEC, which capitalize on the immature immune system of piglets and initiate infection through fimbrial adhesion to intestinal enterocytes, a hallmark mechanism underlying PWD (363–365). Importantly, several isolates in the unified collection, particularly the members of the genera *Escherichia*, *Enterococcus*, *Enterobacter*, and *Intestinimonas*, contained homologs to known virulence-associated genes. Although these annotations are based on sequence homology and should not be interpreted as direct evidence of pathogenicity or *in vivo* virulence potential, these genera have previously been shown to encode virulence-associated factors involved in adhesion, toxin production, secretion systems, and immune modulation with established relevance to intestinal disease (366–368). We therefore retained the annotated virulence factors to capture host-microbe interaction traits and to identify isolates harboring virulence profiles potentially linked to PWD pathogenesis.

Crucially, by linking the functional profiles of isolates in our culture collection to metagenomes from healthy and PWD pigs in an independent dataset, we identified robust associations between genome content and host health. Supervised machine learning models revealed that AMPs, BGCs and ARGs were the strongest predictors of health. The discriminative power of these genetic features across independent datasets highlights the conservation of health-associated genes across swine populations. These findings suggest that microbiome health may depend more on enrichment of biosynthetic and defense functions than on the mere absence of pathogens (369,370). Members of the genera *Prevotella*, *Aneurinibacillus*, *Paratractidigestivibacter*, *Campylobacter*, *Lactobacillus*, and *Phascolarctobacterium* A emerged as dominant contributors to health-associated traits.

Despite these advances, several limitations should be noted. First, while our cultivation approach captured broad diversity, strict anaerobes and fastidious organisms may remain underrepresented. Second, functional annotations rely on available databases and may miss novel activities or strain-specific traits. In addition, feature-specific nucleotide mapping was performed against functional elements derived from a defined cultured isolate collection (SGI and PiBac collection), which may underestimate contributions from highly divergent or uncharacterized strains absent from the reference database. Although this approach preserves

biologically meaningful sequence- and genome-level context, orthologous group- or protein-family-based approaches may provide a complementary framework for broader coverage of community-wide functional potential. Third, health associations were inferred from genome-resolved metagenomic read recruitment rather than direct functional assays or *in vitro* validation. Future work integrating metabolomic, transcriptomic, and gnotobiotic approaches will be essential to confirm the causal role of the identified traits and taxa. In conclusion, this study provides a substantial advance in the genomic and functional characterization of the swine gut microbiome. By generating a deeply curated, genome-resolved isolate collection and identifying potential microbial functional signatures associated with host health status, we establish a scalable framework for mechanistic microbiome research and support future development of culture-based microbiome interventions in livestock systems.

3.6 Declarations

Data availability

Raw sequencing data for the SGI isolates and gut metagenomes from post-weaning diarrhea (PWD)-associated hosts have been deposited in the NCBI Sequence Read Archive (SRA). The metadata associated with the healthy and PWD samples are provided in the **Table S6**. Raw sequences for the SGI isolates are available under BioProject accession PRJNA1389802, while raw gut metagenome sequences from healthy and PWD-associated hosts used for metagenomic read mapping and machine learning model training are available under BioProject accession PRJNA1390059. Metagenomic data from the independent cohort used for machine learning model validation are available under BioProject accession PRJNA1465924. Genomes from the Pig Intestinal Bacterial Collection (PiBac) were downloaded from NCBI SRA under accession PRJNA561470.

Funding

This work was supported by Natural Sciences and Engineering Research Council of Canada (NSERC RGPIN-2023-04359) and Canadian Swine Research and Development Cluster (CSRDC grant # SIP-028-12B) grants awarded to HD.

Code availability

All custom scripts, pipelines and workflows used in the analyses are included in the GitHub repository - PiGICo (https://github.com/zisanurrahman/PiGICo-main_submission/tree/main).

Acknowledgements

We thank the staff at Glenlea Research Station of the University of Manitoba for assistance with the sample collection.

Competing interests

The authors declare that they have no competing interests.

Authors' contribution

HD, AF, and ASMZR conceptualized the study. ASMZR and HD designed the computational framework and performed bioinformatics analyses. ASMZR generated the figures and visualizations and drafted the manuscript. FML conducted the culturomics work, including DNA extraction and library preparation, contributed to bioinformatics analyses, and maintained the SGI isolate collection. HD supervised the project, provided guidance on both experimental and computational framework design, data interpretation, manuscript revision, and secured funding. All authors reviewed, edited, and approved the final manuscript.

4. Chapter 4: General Discussion and Conclusion

4.1 General Discussion

This study provides a substantial expansion of culture-based genomic resources for the swine gut microbiome. By integrating newly generated genomes with existing resources, a unified, high-quality catalog of cultured swine gut bacteria was constructed, significantly extending both taxonomic breadth and functional resolution (274). While PiBac included 110 genomes spanning 40 families and nine phyla (371), the SGI collection generated 300 high-quality non-redundant genomes representing 88 species across 60 genera. Notably, 39% of SGI genomes lacked close matches to PiBac isolates (ANI <95%), including 60 genomes with no detectable close relatives (ANI <80%), indicating that a considerable proportion of the cultured swine gut microbiota remains underrepresented in existing databases.

The SGI collection was dominated by lactobacillaceae and streptococcaceae, clostridiaceae, bacteroidaceae (such as *Prevotella*), consistent with previous sequencing- and cultivation-based studies of the porcine gut microbiome (257,372). In particular, *Lactobacillus* were highly represented, reflecting their ecological prevalence in pigs. *Lactobacillus* species are common members of the porcine small intestine and are adapted to carbohydrate-rich environments and fluctuating oxygen conditions (373). These organisms contribute to

carbohydrate fermentation, organic acid production, pathogen exclusion, and maintenance of intestinal homeostasis, and are frequently associated with resistance to post-weaning enteric disorders (374,375). In addition, the high abundance of *Prevotella* in the porcine gut likely reflects its central role in carbohydrate fermentation (372). *Prevotella* species efficiently degrade plant-derived polysaccharides common in cereal-based swine diets and produce acetate, which can be utilized by butyrate-producing bacteria such as *Clostridium* and *Ruminococcus*. Through these metabolic interactions, *Prevotella* contribute to nutrient utilization, gut barrier function, and intestinal health (372,376).

The taxonomic structure of the pig gut microbiome also differs from that of humans and ruminants due to differences in diet and gastrointestinal physiology. Compared with humans, pigs typically harbor higher abundances of *Lactobacillus* and *Streptococcus*, whereas human gut communities are more enriched in diverse Bacteroidota and Firmicutes and strictly anaerobic polysaccharide degraders (257,377). In contrast to ruminants, where foregut fermentation selects for highly fibrolytic and methanogenic taxa, the pig gut microbiome is enriched in bacteria adapted to starch utilization and rapid nutrient turnover associated with omnivorous feeding behavior (378).

The SGI collection provides experimentally accessible isolates that can support direct investigation of microbial physiology, metabolism, and host-associated functions. Unlike MAG-based approaches, which primarily provide genomic predictions, culture-based resources allow functional validation of microbial traits, assessment of metabolite production, and experimental testing of microbe-microbe or host-microbe interactions under controlled conditions. The use of culturomics in this study also enabled recovery of diverse and previously underrepresented taxa that are often missed by conventional cultivation methods or remain poorly resolved in metagenomic datasets (42,373).

Building on this foundation, the availability of cultured isolates enables genomics-informed discovery of microbiome-derived therapeutics. The present study demonstrates that swine gut bacterial genes encode a wide array of BGCs, metabolic pathways, and antimicrobial functions. Notably, a substantial proportion of identified BGCs showed little or no similarity to previously characterized gene clusters available in public databases, highlighting the swine gut

microbiome as a reservoir of unexplored biosynthetic diversity. Such findings are consistent with growing evidence that host-associated microbiomes harbor unique natural product repertoires shaped by ecological pressures, including microbial competition and host interaction. These biosynthetic features are increasingly recognized as key mediators of colonization resistance, immune modulation, and microbial community stability (379).

Consistent with previous swine gut studies, the swine isolate collection was enriched in RiPP-associated BGCs, including bacteriocin, lantipeptide, and NRPs, which are commonly reported as dominant gut-associated biosynthetic systems due to their roles in microbial competition and colonization resistance (380). In particular, Wang et al (380). reported that bacteriocins, sactipeptides, NRPSs, terpenes, and related RiPP classes are among the most prevalent BGCs in porcine intestinal metagenomes, with marked enrichment across Firmicutes-dominated communities. Similarly, the strong enrichment of RiPPs within Lachnospirales, Clostridiales, Oscillospirales were observed in SGI culture collection. However, studies of the human gut microbiome have shown even broader BGC diversity, with saccharides, RiPPs, NRPSs, PKs, terpenes, and hybrid PKS/NRPS clusters widely distributed across Firmicutes and Ruminococcaceae and *Corynebacterium* (273,275). Donia et al (381). identified more than 14,000 BGCs across human-associated microbial genomes, demonstrating that RiPPs are broadly distributed whereas PKS, NRPS, and hybrid clusters occur across multiple gut-associated lineages, including Firmicutes and Bacteroidota (381). However, the greater diversity of BGCs reported in the human gut microbiome may be partly attributable to the larger number of culturomics studies conducted in humans. These efforts have generated extensive biobanks and isolate genome resources, thereby increasing the capacity to identify and characterize diverse BGC classes compared with the swine gut microbiome. Notably, the large proportion of unassigned GCFs identified in this study suggests that the swine gut harbors substantial unexplored biosynthetic diversity. In addition, our data showed strong AMP enrichment in *Prevotella*, suggesting a more prominent role for Bacteroidota in antimicrobial potential than typically reported in metagenome-based swine studies. Similar observations have been made in the human gut, where peptide-based antimicrobials (especially bacteriocin-like systems and RiPPs) are broadly distributed across both Firmicutes (e.g., Lachnospiraceae, Ruminococcaceae) and Bacteroidota lineages (e.g., *Bacteroides*, *Prevotella*), although Firmicutes remain the dominant producers (381).

The broad yet lineage-specific distribution of metabolic gene clusters observed in this study suggests substantial ecological specialization in primary metabolism and SCFA biosynthesis, consistent with patterns previously reported in the human gut microbiota (382). In particular, genes associated with butyrate production were widely distributed across multiple taxonomic groups, including Lachnospirales, Bacteroidales, and Fusobacteriales. Butyrate is a key SCFA in the swine gut ecosystem, contributing to epithelial integrity, modulation of immune responses, and maintenance of intestinal barrier function. The broad distribution of butyrate-producing pathways across phylogenetically distinct lineages therefore highlights the functional importance of SCFA metabolism within the swine gut microbiome (216). In addition, CAZyme profiling further revealed marked divergence in glycan utilization strategies among microbial lineages. Members of Desulfovibrionaceae, Lactobacillaceae, Bacillaceae, and several additional lineages displayed high proportions of glycoside hydrolases and glycosyltransferases. Expanded CAZyme repertoires likely enhance the capacity of these organisms to degrade structurally diverse dietary and host-derived glycans, thereby increasing metabolic flexibility and competitive fitness in the intestinal environment. In the swine gut microbiota, these carbohydrate-degrading functions are particularly important because they facilitate the conversion of complex polysaccharides into fermentable substrates that support SCFA production (383).

The integration of isolate genomes with metagenomic datasets further demonstrated that functional features such as BGCs and AMPs are strongly associated with clinical diarrhea, suggesting that the contribution of microbiome to gut health partly relies on these functional features rather than presence/absence of certain species. This aligns with emerging models of microbiome resilience, in which diverse biosynthetic and metabolic capabilities contribute to ecosystem stability and resistance to perturbation. In the context of swine production, these insights provide a framework for identifying beneficial taxa and functions that could be targeted for microbiome engineering strategies. Notably, our sequence similarity network analysis revealed that a number of GCFs, particularly those lacking homology to known reference clusters, were significantly depleted in both abundance and richness in PWD-affected piglets. These depleted BGCs spanned several major classes, including NRPS, RiPPs, terpenes, and hybrid clusters. Such BGC classes are commonly associated with gut commensals belonging to

taxa such as Firmicutes (e.g., *Clostridium*, *Lactobacillus*, and related anaerobes) and Actinobacteria, which are well known for producing bioactive secondary metabolites. These compounds play critical roles in shaping microbial community structure; as well as in modulating host physiology and immune responses. The observed depletion of these biosynthetic capacities in PWD suggests a loss of functionally important, metabolite-producing taxa, which may compromise colonization resistance and reduce the overall metabolic resilience of the gut ecosystem.

Beyond individual metabolites, the availability of cultured isolates enables the rational design of defined microbial consortia aimed at improving gut health. In human medicine, microbiota-based interventions such as fecal microbiota transplantation and defined bacterial consortia have shown efficacy in treating disorders such as recurrent *Clostridioides difficile* infection and inflammatory bowel disease (384,385). For example, consortia of commensal Clostridia strains have been shown to promote regulatory T cell differentiation and restore immune homeostasis in murine models of colitis, while defined microbial communities have demonstrated the ability to re-establish colonization resistance and suppress pathogen overgrowth (386). Translating similar strategies to swine production systems could provide sustainable alternatives to antibiotics by enhancing gut resilience, improving feed efficiency, and reducing susceptibility to enteric diseases such as post-weaning diarrhea.

Despite these promising findings, the application of cultured gut bacteria and their metabolites as probiotics or antibiotic alternatives presents both significant advantages and important biological and translational constraints. A major advantage is their ability to mediate colonization resistance through multiple complementary mechanisms, including competitive exclusion for nutrients and adhesion sites, production of antimicrobial compounds (e.g., bacteriocins, microcins, and lantibiotics), and modulation of physicochemical conditions such as luminal pH via short-chain fatty acid production (387,388). For instance, SCFAs such as butyrate not only inhibit the growth of enteric pathogens like *Escherichia coli* and *Salmonella spp.* by acidification but also enhance epithelial barrier integrity through upregulation of tight junction proteins and serve as energy substrates for colonocytes, thereby reinforcing mucosal defense (389,390). In parallel, bacteriocins produced by commensal taxa (e.g., *Lactobacillus*, *Enterococcus*, and *Clostridium spp.*) can exert narrow-spectrum antimicrobial activity,

reducing pathogen burden while preserving commensal diversity which is an advantage over conventional antibiotics that often induce dysbiosis (145,387). Importantly, genome-resolved cultured isolates enable strain-level functional prediction and rational selection, allowing for the identification of specific BGCs, adhesion factors, and metabolic pathways associated with beneficial phenotypes. This facilitates the development of precision probiotics or defined consortia with predictable functional outputs, such as enhanced butyrate production, competitive exclusion of ETEC, or immunomodulation via induction of regulatory T cells (391,392). Such approaches are important in swine systems, where early-life microbiome modulation has been shown to influence susceptibility to PWD and growth performance.

However, several critical limitations complicate their translation into consistent and scalable interventions. One major challenge is ecological engraftment and persistence: introduced strains often fail to stably colonize the gut due to competition with established microbiota, niche saturation, and host-specific selective pressures (393). Even when transient colonization occurs, functional outputs such as metabolite production may be highly context-dependent, varying with diet composition, microbial community structure, and host physiology. Additionally, *in vitro* functional predictions based on genomic data (e.g., presence of BGCs or metabolic pathways) do not always correspond to *in vivo* expression, as many biosynthetic pathways are tightly regulated and condition-dependent (379). Furthermore, large-scale production and application face technological and formulation challenges, including maintaining viability during processing (e.g., freeze-drying), ensuring stability during storage, and achieving effective delivery to the lower gastrointestinal tract without loss of function (394). Finally, the regulatory landscape for live biotherapeutics in food animals remains underdeveloped, with stringent requirements for safety, efficacy, and environmental impact that can limit rapid implementation (395). Moreover, an additional challenge is the therapeutic application of strict anaerobic bacteria, many of which are highly sensitive to oxygen exposure during cultivation, formulation, storage, and delivery. This presents major technical barriers for developing live biotherapeutic products, particularly for taxa derived from the gut microbiome that rapidly lose viability outside anaerobic conditions. Maintaining stability during manufacturing and ensuring survival through feed processing, storage, and gastrointestinal transit therefore remain significant obstacles for large-scale implementation (392,396). Strategies to improve the

delivery of anaerobic live therapeutics include anaerobic encapsulation technologies that protect oxygen-sensitive bacteria during processing and gastrointestinal transit, cryoprotective agents such as trehalose or skim milk used during freeze-drying to preserve cell viability, and spore-based delivery approaches that exploit the natural environmental resistance of spore-forming taxa, particularly members of the Clostridia. Encapsulation matrices composed of alginate, chitosan, or lipid-based carriers have been shown to enhance bacterial survival under aerobic storage conditions and during passage through the acidic upper gastrointestinal tract (397,398). Similarly, optimized cryopreservation and lyophilization protocols can substantially improve long-term stability of strict anaerobes used in microbiome therapeutics (399). However, the scalability and consistency of these approaches under commercial livestock production conditions remain insufficiently explored.

4.2 General conclusion

In conclusion, this study establishes a comprehensive, genome-resolved culture collection of the swine gut microbiome and demonstrates its value for functional and translational research. By combining culturomics with advanced genomic and bioinformatic analyses, it provides a scalable platform for discovering microbiome-derived therapeutics and for developing targeted microbial interventions. These findings pave the way for rational microbiome engineering in livestock, with the potential to enhance animal health, reduce reliance on antibiotics, and support sustainable agricultural practices.

4.2.1 Limitations and Future Directions

Although culturomics substantially expands recoverable diversity, certain fastidious or strictly anaerobic organisms may remain underrepresented due to specific nutritional requirements and limited tolerance to environmental exposure during isolation and cultivation. For example, members of the families Muribaculaceae and Oscillospiraceae are frequently detected in gut metagenomic datasets but are historically difficult to culture because of slow growth and poorly defined metabolic requirements(400). Similarly, highly oxygen-sensitive butyrate producers such as *Faecalibacterium prausnitzii* rapidly lose viability upon oxygen exposure, complicating their recovery and downstream experimental use (401). In addition,

functional predictions are largely based on bioinformatic annotations and require experimental validation to confirm biological activity. The associations observed between microbial functions and host health are also correlative and do not establish causality. Future studies integrating metabolomics, transcriptomics, and controlled *in vivo* models, will be essential to validate the functional roles of candidate strains and metabolites.

From a therapeutic perspective, the diverse biosynthetic potential identified in this culture collection highlights multiple opportunities for microbiome-based interventions in swine health. RiPPs, including bacteriocins, may be particularly attractive candidates because of their potent and often narrow-spectrum antimicrobial activity, which can selectively inhibit pathogens while minimizing disruption to commensal microbiota (145,402). In contrast, NRPs and PKs generally exhibit broader structural and functional diversity, including antimicrobial, immunomodulatory, and signaling activities, but may also present greater challenges related to toxicity, stability, and large-scale production (167,181,215).

Therapeutic applications could involve either the direct administration of live bacterial strains or the use of purified microbial metabolites. Live biotherapeutic strains may provide sustained metabolite production, competitive exclusion of pathogens, and long-term ecological integration within the gut microbiome (392). Conversely, purified bacteriocins or other secondary metabolites may offer greater dosing precision, safety control, and regulatory standardization while avoiding risks associated with bacterial colonization or horizontal gene transfer (403). The choice between these approaches will likely depend on the stability of colonization, metabolite bioavailability, host specificity, and safety profiles of individual strains and compounds.

Future research should therefore focus on functional validation of predicted BGCs, characterization of metabolite expression under *in vivo* conditions, and assessment of antimicrobial activity against major swine pathogens. Additional work integrating metabolomics, transcriptomics, and *in vivo* animal studies will be necessary to determine whether these candidate probiotics and their metabolites can improve gut health, pathogen resistance, and production performance in commercial swine systems. Furthermore, optimizing

cultivation methods for fastidious anaerobes and improving scalable delivery systems will be essential for translating these findings into practical therapeutic applications.

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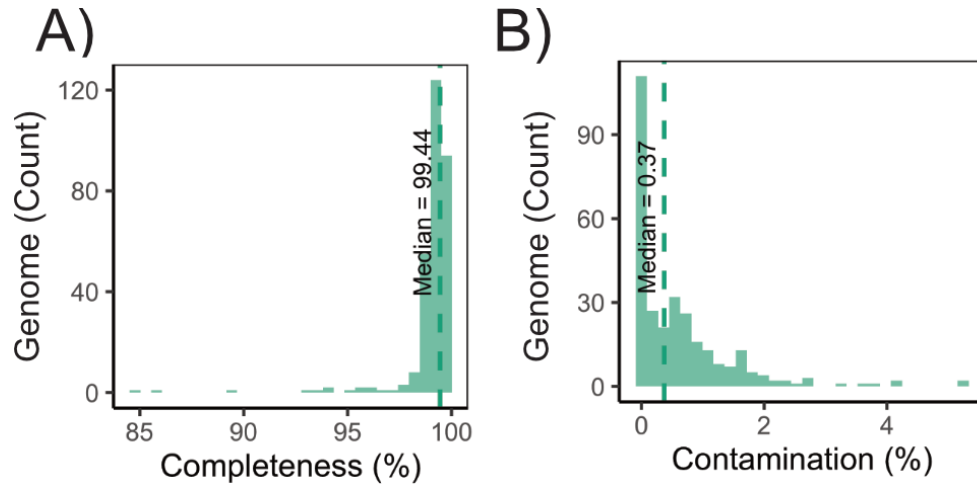
6. Appendix A: Supplementary Tables

Supplementary Table S1: Sample metadata, taxonomic classification (GTDB), and assembly stats for the SGI isolates. Available at: <https://doi.org/10.6084/m9.figshare.33166925>

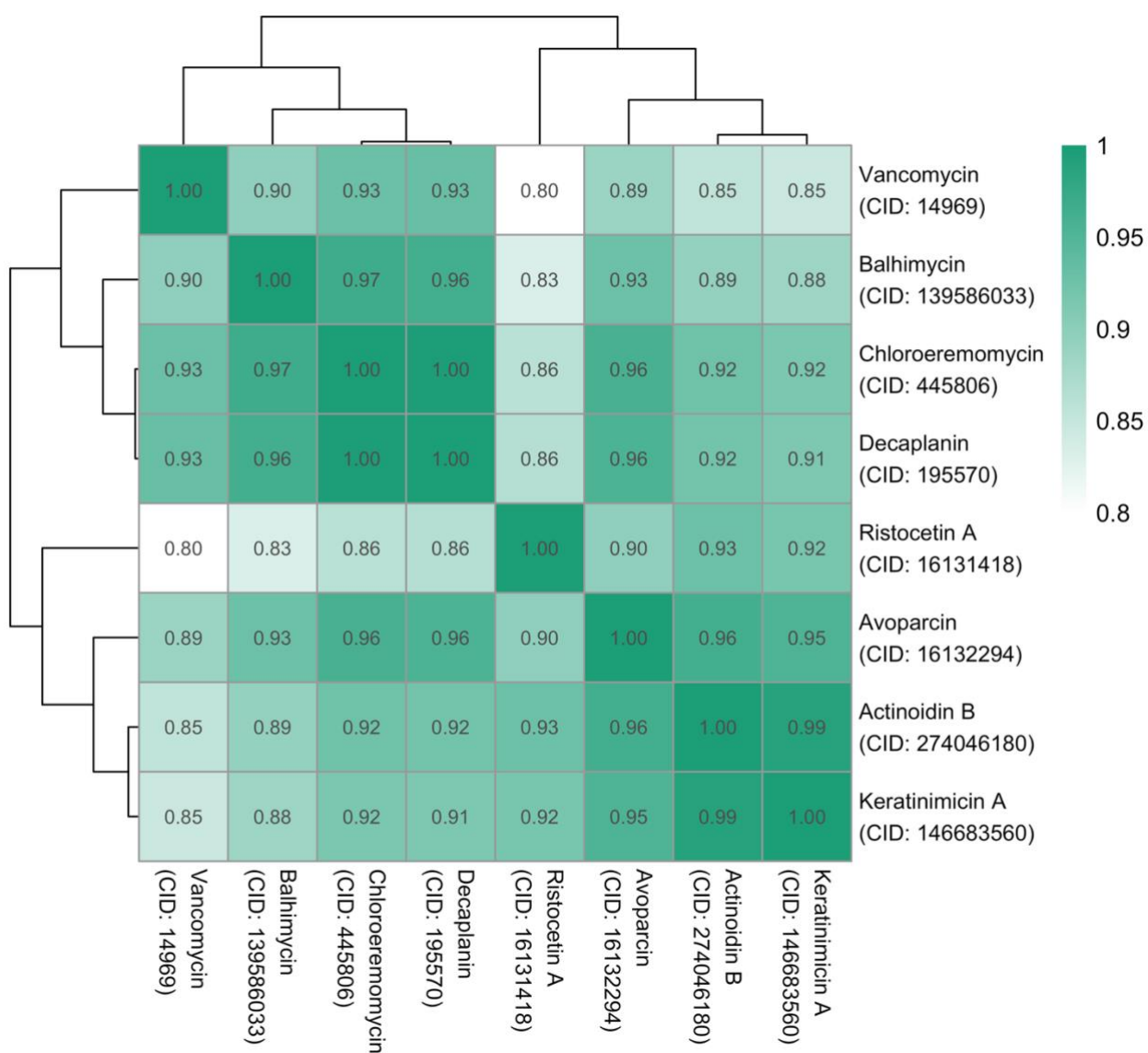
Supplementary Table S2: Overview of sample sources, study components, and downstream applications. Available at: <https://doi.org/10.6084/m9.figshare.33166925>

Supplementary Table S3: Metadata related to the metagenomic samples used in this study. Available at: <https://doi.org/10.6084/m9.figshare.33166925>

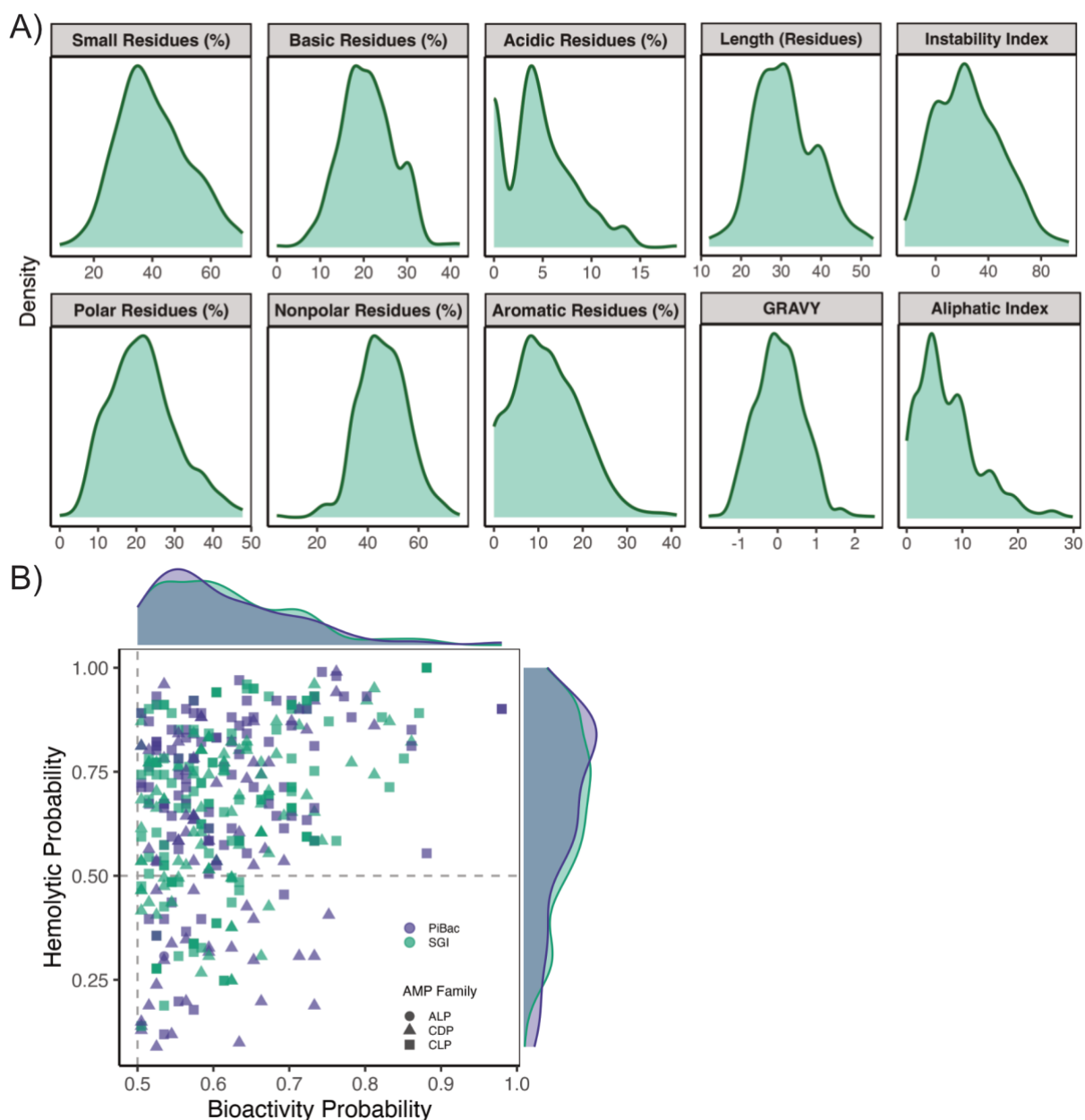
7. Appendix B: Supplementary Figures



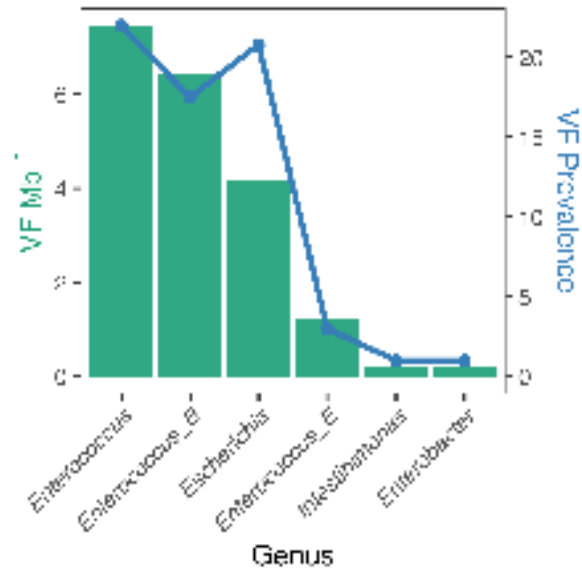
Supplementary Figure S1. Genome quality metrics of the SGI isolate collection. A) Distribution of estimated genome percent completeness (A), and contamination (B) across the SGI genomes.



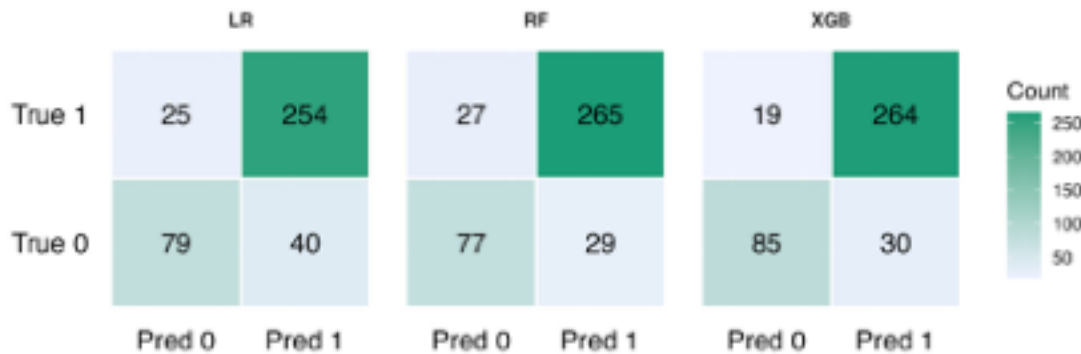
Supplementary Figure S2. Pairwise Tanimoto similarity matrix of selected MIBiG reference compounds (Related to Figure 3A). Color gradient from white to green represents increasing structural similarity. Hierarchical clustering groups compounds based on Euclidean distance. PubChem CID for each compound is provided in parenthesis.



Supplementary Figure S3. Characterization of antimicrobial peptides (AMPs) identified in swine gut bacterial genomes. **A)** Distribution of physicochemical properties among predicted AMPs, including residue composition (e.g., small, basic, acidic), peptide length, instability index, polarity, GRAVY (hydropathy index), and aliphatic/aromatic content. Density plots show the diversity of AMP chemical features across all detected sequences. **B)** Functional landscape of AMPs based on predicted bioactivity and hemolytic potential. Density plots on the margins indicate distribution trends across the two predicted probabilities.



Supplementary Figure S4. Virulence factor distribution across the swine gut isolates. Bar and line chart depicting virulence factor (VF) counts per genome (left y-axis) and VF prevalence across genomes within each genus (right y-axis). VF annotations were assigned using VFDB.



Supplementary Figure S5. Confusion matrices for each classifier showing predicted vs. true labels. Color intensity reflects sample counts.