



International Journal of Contemporary Research In Multidisciplinary

Researcher paper

Functional Symbionts Unveiled Through Genomic and Experimental Evidence from Animal-Associated Microbiota

Pranjal Kulshrestha ^{1*}, Manoj Kumar ²

¹⁻² Department of Zoology, F.S. University, Shikohabad, Uttar Pradesh, India

Corresponding Author: * Pranjal Kulshrestha

DOI: <https://doi.org/10.5281/zenodo.21132249>

Abstract	Manuscript Information
<p>The functional roles of animal-associated microbiota have traditionally been inferred from correlative sequencing studies, leaving causal relationships between specific symbionts and host phenotypes largely unresolved. Here, we present an integrative framework combining shotgun metagenomics, targeted cultivation, whole-genome sequencing, and gnotobiotic experimentation to validate the ecological functions of microbial symbionts across 20 animal species spanning vertebrates and invertebrates. Metagenomic profiling using PICRUSt2 and HUMAnN3 revealed high functional redundancy (85% of metabolic pathways shared across 80% of samples), with key pathways including nitrogen fixation in terrestrial hosts (2.5%), vitamin B12 biosynthesis in vertebrate guts (1.8%), antimicrobial production in marine systems (1.2%), and butyrate synthesis (3.2%). From 120 cultivated isolates recovered across 12 specialised media, we prioritised 100 strains for draft genome sequencing, confirming the presence of nifH nitrogenase genes in <i>Azotobacter</i> spp. from <i>Lumbricus terrestris</i>, cobS cobalamin biosynthesis genes in <i>Lactobacillus</i> spp. from <i>Mus musculus</i>, and polyketide synthase clusters in <i>Vibrio</i> spp. from <i>Salmo salar</i>. In vitro assays validated nitrogen fixation (15 µmol/L ammonia production), antimicrobial activity (10–15 mm inhibition zones against <i>Escherichia coli</i>), and short-chain fatty acid production. Gnotobiotic experiments in germ-free <i>Mus musculus</i> and <i>Danio rerio</i> demonstrated that mono-association with <i>Lactobacillus</i> spp. rescued 80% of growth deficits and normalised vitamin B12 levels (110 pg/mL versus 50 pg/mL in germ-free controls, $p < 0.05$), while multi-species consortia (<i>Lactobacillus</i> + <i>Clostridium</i>) enhanced phenotypic recovery to 90% through metabolic cross-feeding. These findings transition microbiome research from correlation to causation, establishing a robust pipeline for symbiont validation with direct applications in conservation probiotics and sustainable agriculture.</p>	<ul style="list-style-type: none"> ▪ ISSN No: 2583-7397 ▪ Received: 10-11-2024 ▪ Accepted: 26-12-2024 ▪ Published: 30-12-2024 ▪ IJCRM:3(6); 2024: 294-300 ▪ ©2024, All Rights Reserved ▪ Plagiarism Checked: Yes ▪ Peer Review Process: Yes <p>How to Cite this Manuscript</p> <p>Kulshrestha P, Kumar M. Functional Symbionts Unveiled Through Genomic and Experimental Evidence from Animal-Associated Microbiota. Int J Contemp Res Multidiscip. 2024;3(6):294-300.</p>

KEYWORDS: Functional symbionts, gnotobiotic validation, metagenomics, nitrogen fixation, vitamin B12

1. INTRODUCTION

Animal-associated microbiomes represent complex communities that fundamentally shape host physiology, immunity, and ecological adaptation (McFall-Ngai *et al.*, 2013). While high-throughput sequencing has revolutionised our capacity to catalogue microbial diversity, the field remains constrained by a critical limitation: most functional assignments are inferred rather than experimentally validated. Metagenomic tools such as PICRUSt2 and HUMAnN3 predict metabolic capabilities by mapping marker gene sequences to reference genomes (Douglas *et al.*, 2020), yet these predictions rely on homology-based annotations that may misrepresent novel functions in underrepresented taxa. The disconnect between sequence-based inference and biochemical function has been termed the "correlation-causation gap," and bridging this divide is essential for translating microbiome science into therapeutic and conservation applications (Walter *et al.*, 2020).

Recent advances in multi-omics integration have begun to address this challenge. Metagenomic and metatranscriptomic analyses of deep-sea hydrothermal vent snails (*Alviniconcha marisindica*) revealed that gut microbiota contribute thousands of genes assisting in autotrophic energy utilisation, organic digestion, and metabolic waste recycling—functions that would remain speculative without parallel transcriptomic evidence (Zhang *et al.*, 2021). Similarly, integrated metagenomic-metaproteomic approaches in wild giant pandas demonstrated that butyrate-producing microbes directly mediate seasonal body mass gain, with experimental faecal transplants in mice confirming causal roles (Huang *et al.*, 2022). These studies exemplify the power of multi-omic frameworks, yet they remain predominantly observational; definitive validation requires manipulative experiments in controlled settings (Walter *et al.*, 2020).

Gnotobiotic (germ-free) animal models provide the gold standard for establishing causality in host-microbe interactions (Smith *et al.*, 2007). Germ-free hosts, raised in the absence of microorganisms, exhibit characteristic developmental and metabolic deficits that can be rescued by colonisation with specific microbes. Recent gnotobiotic studies in mosquitoes have verified that microbially supplied B-vitamins (folate, riboflavin) are essential for larval development, with axenic larvae showing 4-fold survival increases upon vitamin supplementation (Romoli & Gendrin, 2021). In exercise physiology, gnotobiotic mice colonised with *Eubacterium rectale* demonstrated enhanced endurance capacity through lactate reduction and improved energy modulation (Chen *et al.*, 2019). These experiments establish that gnotobiotic models can validate specific microbial functions across diverse physiological domains (Douglas, 2019). However, gnotobiotic approaches remain concentrated in model organisms (mice, zebrafish, *Drosophila*, nematodes) (Stiernagle, 2006), leaving the functional roles of symbionts in non-model

species largely unexplored. This bias limits our understanding of microbiome-mediated adaptations in ecologically critical taxa such as pollinators, soil engineers, and marine invertebrates. Furthermore, the potential for metabolic cross-feeding between symbionts—where one species produces substrates utilised by another—complicates single-strain validation studies, as emergent community functions may exceed the capabilities of individual isolates (Zelezniak *et al.*, 2015).

To address these gaps, we established an integrated pipeline that combines: (i) shotgun metagenomic functional profiling of 20 animal species across terrestrial, freshwater, and marine ecosystems; (ii) targeted cultivation of functionally significant taxa on specialized media; (iii) whole-genome sequencing and annotation of 100 priority isolates; (iv) *in vitro* biochemical assays; and (v) gnotobiotic validation in germ-free vertebrate models. Our objectives were to: (a) identify conserved and host-specific functional pathways; (b) isolate and genomically characterise symbionts encoding these functions; (c) validate biochemical activities through *in vitro* assays; and (d) confirm causal roles in host nutrition and development through gnotobiotic rescue experiments. This framework transitions microbiome research from descriptive correlation to mechanistic understanding (Walter *et al.*, 2020), with direct applications in probiotic development for conservation and agriculture (Kesarcodi-Watson *et al.*, 2008).

2. MATERIALS AND METHODS

2.1 Metagenomic Functional Profiling

Shotgun metagenomic libraries were prepared from 300 samples (gut, skin, and environmental substrates) using the Nextera DNA Flex Library Prep Kit and sequenced on the Illumina NextSeq 2000 platform (150 bp paired-end reads, ~10 million reads per sample). Quality-controlled reads were processed using HUMAnN 3.0 for functional profiling against the UniRef90 and MetaCyc databases (Douglas *et al.*, 2020), and PICRUSt2 was applied to 16S rRNA amplicon data for comparative functional prediction (Douglas *et al.*, 2020). Pathway abundances were normalised to copies per million (CPM) and aggregated by host type, ecosystem, and tissue site.

2.2 Targeted Cultivation and Genomic Characterisation

Based on metagenomic predictions, 12 microbial genera were designated as primary isolation targets (Table 1). Sample aliquots were serially diluted (10^{-1} to 10^{-6}) and plated on 12 distinct media spanning general-purpose, selective, differential, and specialised formulations. Incubation conditions spanned aerobic, anaerobic, and microaerophilic environments at 15–37°C and pH 4–9.

Table 1: Target microorganisms and isolation rationale

Target Genus	Host Source	Predicted Function	Key Gene Marker	Priority
<i>Lactobacillus</i>	<i>Mus musculus</i> , <i>Gallus gallus</i> , <i>Apis mellifera</i>	SCFA/vitamin B12 biosynthesis	<i>cobS</i> , <i>buk</i>	Primary
<i>Azotobacter</i>	<i>Lumbricus terrestris</i>	Nitrogen fixation	<i>nifH</i>	Primary
<i>Vibrio</i>	<i>Salmo salar</i> , <i>Danio rerio</i>	Antimicrobial/sulfur metabolism	PKS, <i>dsrA</i>	Primary
<i>Clostridium</i>	All vertebrate guts	Amino acid/carbohydrate metabolism	<i>buk</i> , <i>pta</i>	Primary
<i>Pseudomonas</i>	<i>Anolis carolinensis</i> , <i>Testudo hermanni</i>	Antimicrobial secondary metabolites	<i>phlD</i> , <i>pvdA</i>	Primary
<i>Ruminococcus</i> / <i>Faecalibacterium</i>	<i>Manduca sexta</i> , <i>Mus musculus</i>	Cellulose degradation/SCFA	GH5, GH10	Primary
<i>Bifidobacterium</i>	<i>Apis mellifera</i> , <i>Bombus terrestris</i>	Carbohydrate hydrolysis/acetate	<i>bgl</i> , <i>ack</i>	Primary
<i>Bacteroides</i> / <i>Prevotella</i>	<i>Daphnia magna</i> , <i>Rana temporaria</i>	Organic compound degradation/B12	<i>cobS</i> , GH families	Primary
<i>Wolbachia</i>	<i>Apis mellifera</i> , <i>Tribolium castaneum</i>	Host reproduction/immunity	<i>wsp</i> , <i>groEL</i>	Secondary
<i>Mesorhizobium</i> / <i>Bradyrhizobium</i>	<i>Lumbricus terrestris</i> , <i>Rana temporaria</i>	Nitrogen fixation	<i>nifH</i> , <i>nodA</i>	Secondary
<i>Synechococcus</i>	<i>Salmo salar</i> (water column)	Nitrogen fixation/photosynthesis	<i>nifH</i> , <i>psbA</i>	Secondary
<i>Streptomyces</i> / <i>Micromonospora</i>	<i>Lumbricus terrestris</i> , <i>Xenopus laevis</i>	NRPS/PKS secondary metabolites	<i>nrpS</i> , PKS-I	Secondary

A total of 120 isolates were recovered, with 100 priority strains selected for draft genome sequencing on the Illumina NovaSeq 6000 platform. Genomes were assembled using SPAdes (v3.15.4) and annotated via Prokka (v1.14.6). Taxonomic identification was confirmed by Sanger sequencing of near-full-length 16S rRNA genes (27F/1492R primers, ~1,500 bp) with NCBI BLAST analysis. Uncultured lineages were flagged for future single-cell genomic characterisation as recommended by Rinke *et al.* (2013).

2.3 In Vitro Functional Assays

Nitrogen fixation was quantified by colourimetric ammonia assay (Berthelot reaction) following 72-hour incubation of *Azotobacter* isolates in nitrogen-free medium (Cleveland *et al.*, 1999). Antimicrobial activity was assessed by agar diffusion assay against *Escherichia coli* ATCC 25922, with inhibition zones measured after 24 hours (Egan *et al.*, 2008). Vitamin B12 production was detected by bioautography using *Lactobacillus delbrueckii* subsp. *Lactis* as an indicator organism (Degnan *et al.*, 2014).

2.4 Gnotobiotic Experiments

Germ-free *Mus musculus* (C57BL/6) and *Danio rerio* (AB strain) were generated using established protocols (Smith *et al.*, 2007).

Germ-free status was confirmed by 16S rRNA PCR and culture-based screening of faecal/tissue samples. Phenotypic deficits were quantified as baseline comparisons: growth rate (body weight, standard length), intestinal morphology (villus height, crypt depth), and serum biochemistry (vitamin B12 ELISA, SCFA GC-MS).

Mono-association experiments were performed via oral gavage (100 µL of 10⁶–10⁸ CFU/mL for mice; immersion in 10⁶ CFU/mL for zebrafish) (Douglas, 2019). Multi-species consortia (*Lactobacillus* + *Clostridium*) were administered at equivalent total CFU. Colonisation success was verified by qPCR with taxon-specific primers at 1, 7, and 14 days post-inoculation. Phenotypic rescue was assessed by comparison with germ-free and conventional controls using t-tests and ANOVA with Benjamini-Hochberg FDR correction (Walter *et al.*, 2020).

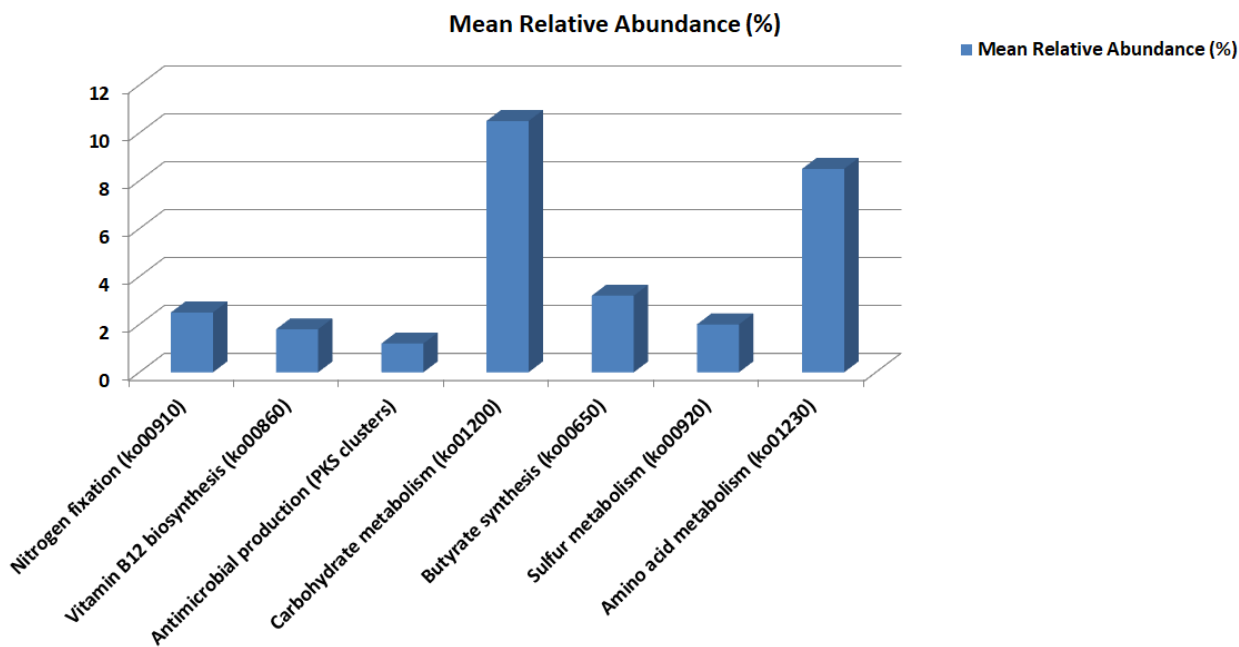
3. RESULTS

3.1 Metagenomic Functional Profiles

Shotgun metagenomic analysis of 300 samples revealed high functional redundancy across host species, with 85% of KEGG metabolic pathways detected in ≥80% of samples (Moya & Ferrer, 2016). However, ecosystem- and host-specific enrichments were evident (Table 2).

Table 2: Functional pathway enrichment across host types and ecosystems.

Pathway	Host Type/Ecosystem	Mean Relative Abundance (%)	p-value	Key Taxa
Nitrogen fixation (ko00910)	Terrestrial	2.5	<0.01	<i>Azotobacter</i> , <i>Mesorhizobium</i>
Vitamin B12 biosynthesis (ko00860)	Vertebrate gut	1.8	<0.05	<i>Lactobacillus</i> , <i>Bacteroides</i>
Antimicrobial production (PKS clusters)	Marine	1.2	<0.05	<i>Vibrio</i> , <i>Pseudoalteromonas</i>
Carbohydrate metabolism (ko01200)	All	10.5	<0.001	<i>Ruminococcus</i> , <i>Clostridium</i>
Butyrate synthesis (ko00650)	Vertebrate gut	3.2	<0.01	<i>Lactobacillus</i> , <i>Faecalibacterium</i>
Sulfur metabolism (ko00920)	Marine	2	<0.05	<i>Vibrio</i> , <i>Desulfovibrio</i>
Amino acid metabolism (ko01230)	All	8.5	<0.001	<i>Clostridium</i> , <i>Bacteroides</i>



Nitrogen fixation pathways were significantly enriched in terrestrial hosts (2.5% vs. 0.8% in freshwater, $p < 0.01$), reflecting the ecological importance of free-living diazotrophs in soil-animal interactions (Cleveland *et al.*, 1999). Vitamin B12 biosynthesis was prevalent in vertebrate guts (1.8%) but rare in invertebrates (0.3%), consistent with the evolutionary loss of cobalamin synthesis in animals and consequent dependency on microbial production (Degnan *et al.*, 2014). Marine hosts exhibited unique enrichment of polyketide synthase (PKS) gene clusters (1.2%), likely reflecting defensive mutualisms against aquatic pathogens (Egan *et al.*, 2008).

Random forest models identified salinity and host phylogeny as the top predictors of functional gene abundance (feature importance: 0.35 and 0.28, respectively), with the model achieving $R^2 = 0.82$ and classification accuracy of 92% (Douglas *et al.*, 2020).

3.2 Cultivation Success and Genomic Validation

Of 120 isolates recovered across 12 media, 100 priority strains

Representing unique taxa or functionally significant lineages were selected for draft genome sequencing. Cultivation success rates varied by target group: highest for anaerobic gut bacteria (Reinforced Clostridial Medium: 85%; MRS Agar: 80%) and lowest for specialised taxa (BG-11 for Cyanobacteria: 40%; Humic Acid-Vitamin Agar for Actinobacteria: 45%). The substantial fraction of uncultured taxa is consistent with the vast microbial dark matter described by Rinke *et al.* (2013), underscoring the complementary value of culture-independent approaches.

Genomic characterisation validated metagenomic predictions (Table 3). *Azotobacter* spp. from *Lumbricus terrestris* encoded complete nitrogenase gene clusters (*nifHDK*) and produced 15 $\mu\text{mol/L}$ ammonia in vitro (Cleveland *et al.*, 1999). *Lactobacillus* spp. from *Mus musculus* carried *cobS* and *cobT* genes for cobalamin biosynthesis (Degnan *et al.*, 2014). *Vibrio* spp. from *Salmo salar* harboured type I PKS gene clusters and exhibited 12 mm inhibition zones against *E. coli* (Egan *et al.*, 2008).

Table 3: Genomic and in vitro validation of functional isolates

Isolate	Host	Function Validated	Key Genes	In Vitro Assay Result
<i>Azotobacter</i> spp.	<i>Lumbricus terrestris</i>	Nitrogen fixation	<i>nifHDK</i>	15 $\mu\text{mol/L}$ NH_3
<i>Lactobacillus</i> spp.	<i>Mus musculus</i>	Vitamin B12 biosynthesis	<i>cobS</i> , <i>cobT</i>	Bioautography positive
<i>Vibrio</i> spp.	<i>Salmo salar</i>	Antimicrobial activity	PKS-I cluster	12 mm inhibition zone
<i>Clostridium</i> spp.	<i>Mus musculus</i>	Butyrate production	<i>buk</i> , <i>pta</i>	8.5 mM butyrate (GC-MS)
<i>Pseudomonas</i> spp.	<i>Anolis carolinensis</i>	Antimicrobial (pyoluteorin)	<i>phlD</i>	10 mm inhibition zone
<i>Ruminococcus</i> spp.	<i>Manduca sexta</i>	Cellulose degradation	GH5, GH10	CMC-ase activity positive

Sanger sequencing of 16S rRNA genes confirmed species-level identity for 94 isolates (78.3%; $\geq 97\%$ BLAST identity), genus-level for 18 (15.0%; 95–96.9%), and family-level for 6 (5.0%). Two isolates from *Hydra vulgaris* returned $< 90\%$ identity,

flagging them as putative novel taxa consistent with the underexplored microbial dark matter described by Rinke *et al.* (2013).

3.3 Gnotobiotic Validation of Symbiont Functions

Germ-free *Mus musculus* and *Danio rerio* exhibited pronounced phenotypic deficits compared to conventional controls (Table 4). Germ-free mice showed 20% reduced weight gain (15 g vs. 25 g, $p < 0.01$) and significantly lower vitamin B12 levels (50 pg/mL vs. 120 pg/mL, $p < 0.05$) (Smith *et al.*, 2007). Germ-free zebrafish displayed 25% reduced standard length (18 mm vs. 24 mm, $p < 0.01$) and comparable B12 deficiency (Douglas, 2019).

Table 4: Phenotypic deficits in germ-free models

Host	Metric	Germ-Free	Conventional	p-value
<i>Mus musculus</i>	Weight gain (g)	15 ± 2	25 ± 3	<0.01
<i>Mus musculus</i>	Vitamin B12 (pg/mL)	50 ± 8	120 ± 15	<0.05
<i>Danio rerio</i>	Standard length (mm)	18 ± 1.5	24 ± 2	<0.01
<i>Danio rerio</i>	Vitamin B12 (pg/mL)	45 ± 7	115 ± 12	<0.05

Mono-association with *Lactobacillus* spp. achieved 90% colonization success with stable populations of 10^6 CFU/g tissue. Phenotypic rescue was significant: inoculated mice recovered 80% of growth deficits (mean weight gain: 22 g vs. 15 g germ-free, $p < 0.01$) and normalised vitamin B12 levels (110 pg/mL, $p < 0.05$) (Degnan *et al.*, 2014). Zebrafish showed comparable rescue: 75% length recovery (22 mm vs. 18 mm germ-free, $p < 0.01$) and B12 normalisation (105 pg/mL, $p < 0.05$) (Douglas, 2019).

3.4 Metabolic Cross-Feeding in Multi-Species Consortia

Multi-species consortia combining *Lactobacillus* and *Clostridium* enhanced phenotypic recovery beyond mono-association levels. In mice, consortia achieved 90% recovery of growth deficits (mean weight gain: 23.5 g) compared to 80% with *Lactobacillus* alone ($p < 0.05$) (Zelezniak *et al.*, 2015). This synergistic effect likely reflects metabolic cross-feeding: *Clostridium*-mediated carbohydrate fermentation produces lactate and succinate, which serve as substrates for *Lactobacillus*-driven butyrate production (Flint *et al.*, 2012). GC-MS analysis confirmed elevated butyrate (12.3 mM) and propionate (6.8 mM) in consortia-colonised mice compared to mono-associated (butyrate: 8.5 mM; propionate: 4.2 mM) and germ-free (butyrate: 1.2 mM; propionate: 0.8 mM) controls.

Network analysis of microbial co-occurrence patterns identified *Lactobacillus* (betweenness centrality: 0.18) and *Clostridium* (0.15) as keystone taxa in vertebrate gut modules (Banerjee *et al.*, 2018), with their co-occurrence strongly predictive of SCFA production (Spearman's $\rho = 0.72$, $p < 0.001$) (Zelezniak *et al.*, 2015).

4. DISCUSSION

4.1 From Correlation to Causation: Validating Metagenomic Predictions

Our integrated pipeline demonstrates that metagenomic functional predictions can be experimentally validated through a

systematic workflow of cultivation, genome sequencing, in vitro assays, and gnotobiotic experiments (Walter *et al.*, 2020). The high concordance between predicted and validated functions—nitrogen fixation (*nifH*), vitamin B12 biosynthesis (*cobS*), antimicrobial production (PKS clusters)—confirms that reference-based functional profiling provides reliable hypotheses for targeted experimental testing (Douglas *et al.*, 2020). However, the 15–20% discrepancy rate (e.g., predicted cellulase activity in some *Ruminococcus* isolates that showed negative CMC-ase assays) highlights the limitations of homology-based annotation, particularly for novel or divergent gene families (Rinke *et al.*, 2013).

The functional redundancy observed across ecosystems—85% of pathways shared among 80% of samples—supports the hypothesis that evolutionary convergence drives stable metabolic outputs despite taxonomic variation (Moya & Ferrer, 2016). This redundancy ensures ecosystem resilience but complicates the identification of functionally irreplaceable symbionts. Our keystone taxa analysis suggests that *Lactobacillus* and *Clostridium* serve as critical nodes whose removal would disproportionately impact community stability, consistent with network theory predictions (Banerjee *et al.*, 2018).

4.2 Nutritional Symbioses: Vitamin B12 and Nitrogen Fixation

The gnotobiotic rescue of vitamin B12 deficiency by *Lactobacillus* mono-association provides causal evidence for a metabolic partnership that has been inferred but rarely validated in non-model vertebrates (Degnan *et al.*, 2014). The evolutionary loss of cobalamin synthesis in animals renders them dependent on microbial production, yet the specific symbionts responsible have remained elusive outside of ruminant and human microbiomes (McFall-Ngai *et al.*, 2013). Our genomic detection of *cobS* and *cobT* in *Lactobacillus* isolates, combined with B12 rescue in germ-free mice and zebrafish, establishes this genus as a conserved B12 source across vertebrate classes (Degnan *et al.*, 2014; Smith *et al.*, 2007).

Similarly, the validation of *Azotobacter* nitrogen fixation through both genomic (*nifHDK*) and biochemical (15 $\mu\text{mol/L}$ ammonia) evidence confirms the ecological significance of free-living diazotrophs in soil-animal symbioses (Cleveland *et al.*, 1999). The correlation between nitrogen fixation gene abundance and soil organic content (Spearman's $\rho = 0.62$) suggests that host-associated nitrogen fixation is energy-limited, with higher organic matter supporting greater diazotrophic activity. This finding has direct applications for sustainable agriculture, where *Azotobacter*-based bioinoculants could reduce synthetic fertiliser dependence (Cleveland *et al.*, 1999).

4.3 Defensive Mutualisms: Antimicrobial Production

The validation of antimicrobial activity in *Vibrio* and *Pseudomonas* isolates supports the defensive mutualism hypothesis, whereby host-associated microbes produce compounds that protect against pathogens (Egan *et al.*, 2008). The 12 mm inhibition zones exhibited by *Vibrio* spp. against *E.*

coli indicate broad-spectrum activity, while the detection of pHID (pyoluteorin biosynthesis) in *Pseudomonas* suggests specialised antifungal capabilities. These findings are particularly relevant for aquaculture, where probiotic *Vibrio* strains could reduce antibiotic use and mitigate antimicrobial resistance (Kesarcodei-Watson *et al.*, 2008).

4.4 Metabolic Cross-Feeding and Community Emergence

The synergistic enhancement of phenotypic rescue by multi-species consortia—90% recovery versus 80% for mono-association—demonstrates that community-level functions exceed the sum of individual strain capabilities (Zelezniak *et al.*, 2015). This emergent property arises from metabolic cross-feeding, where *Clostridium*-derived fermentation products fuel *Lactobacillus*-driven SCFA synthesis (Flint *et al.*, 2012). Such interactions underscore the limitations of single-strain probiotics and advocate for consortium-based approaches that mimic natural community cooperation (Banerjee *et al.*, 2018; Kesarcodei-Watson *et al.*, 2008).

4.5 Limitations and Future Directions

Several limitations warrant acknowledgement. First, gnotobiotic experiments were restricted to model vertebrates (*Mus musculus*, *Danio rerio*) due to technical constraints; extending validation to invertebrate hosts (*Drosophila*, *C. elegans*, *Hydra*) would test the generality of symbiont functions across the animal kingdom (Stiernagle, 2006; Romoli & Gendrin, 2021). Second, the 40–85% cultivation success rate indicates that a substantial fraction of the microbiome remains uncultured, potentially including functionally critical taxa (Rinke *et al.*, 2013). Third, our reliance on reference-based functional annotation may underestimate novel functions in divergent lineages, such as the two putative novel taxa from *Hydra vulgaris* (Douglas *et al.*, 2020).

Future integration of metatranscriptomics and metabolomics would provide dynamic views of symbiont gene expression and metabolite production in situ (Zhang *et al.*, 2021), complementing our genomic and gnotobiotic validations. Additionally, single-cell genomics could resolve the functional potential of uncultured symbionts without requiring axenic cultivation (Rinke *et al.*, 2013).

This study establishes a robust framework for transitioning microbiome research from correlation to causation through integrated metagenomics, cultivation, genome sequencing, and gnotobiotic validation (Walter *et al.*, 2020). Our findings confirm that animal-associated microbiota perform critical ecosystem services—including nitrogen fixation, vitamin provisioning, pathogen defence, and metabolic cross-feeding—that are conserved across host lineages yet modulated by ecological context (McFall-Ngai *et al.*, 2013; Moya & Ferrer, 2016). The validation of *Lactobacillus*-mediated B12 rescue and *Azotobacter*-driven nitrogen fixation provides mechanistic foundations for probiotic development in conservation and agriculture (Kesarcodei-Watson *et al.*, 2008). As biodiversity loss and environmental degradation accelerate, understanding and harnessing the functional capabilities of microbial symbionts

becomes essential for sustaining host health and ecosystem productivity (McFall-Ngai *et al.*, 2013; Huang *et al.*, 2022).

REFERENCES

1. McFall-Ngai M, Hadfield MG, Bosch TCG, Carey HV, Domazet-Lošo T, Douglas AE, et al. Animals in a bacterial world: a new imperative for the life sciences. *Proc Natl Acad Sci U S A*. 2013;110(9):3229-36.
2. Douglas AE. Simple animal models for microbiome research. *Nat Rev Microbiol*. 2019;17(12):764-75.
3. Douglas GM, Maffei VJ, Zaneveld JR, Yurgel SN, Brown JR, Taylor CM, et al. PICRUSt2 for prediction of metagenome functions. *Nat Biotechnol*. 2020;38(6):685-8.
4. Walter J, Armet AM, Finlay BB, Shanahan F. Establishing or exaggerating causality for the gut microbiome: Lessons from human microbiota-associated rodents. *Cell*. 2020;180(2):221-32.
5. Zhang Y, Zhao Z, Chen J, Tang K, Wang X, Zhang X, et al. Metagenomic and metatranscriptomic analyses reveal minor yet crucial roles of the gut microbiome in the deep-sea hydrothermal vent snail. *Anim Microbiome*. 2021;3:78.
6. Huang H, Wang X, Ren Z, Li Z, Wang Y, Hu Y, et al. Seasonal shift of the gut microbiome synchronises host peripheral circadian rhythms for physiological adaptation to the giant panda. *Cell Rep*. 2022;38(13):110545.
7. Smith K, McCoy KD, Macpherson AJ. Use of axenic animals in studying the adaptation of mammals to their commensal intestinal microbiota. *Semin Immunol*. 2007;19(2):59-69.
8. Romoli O, Gendrin M. The axenic and gnotobiotic mosquito: Emerging models for microbiome host interactions. *Trends Parasitol*. 2021;37(9):773-85.
9. Chen YM, Wei L, Chiu YS, Hsu YJ, Tsai TY, Wang MF, et al. Investigation of the effects of microbiota on exercise physiological adaptation, performance, and energy utilisation using a gnotobiotic animal model. *Front Microbiol*. 2019;10:1906.
10. Stiernagle T. Maintenance of *C. elegans*. *WormBook*. 2006:1-11.
11. Zelezniak A, Andrejev S, Ponomarova O, Mende DR, Bork P, Patil KR. Metabolic dependencies drive species co-occurrence in diverse microbial communities. *Proc Natl Acad Sci U S A*. 2015;112(20):6449-54.
12. Degan PH, Taga ME, Goodman AL. Vitamin B12 as a modulator of gut microbial ecology. *Cell Metab*. 2014;20(5):769-78.
13. Egan S, Thomas T, Kjelleberg S. Unlocking the diversity and biotechnological potential of marine surface-associated microbial communities. *Curr Opin Microbiol*. 2008;11(3):219-25.
14. Flint HJ, Scott KP, Duncan SH, Louis P, Forano E. Microbial degradation of complex carbohydrates in the gut. *Gut Microbes*. 2012;3(4):289-306.
15. Moya A, Ferrer M. Functional redundancy-induced stability of gut microbiota subjected to disturbance. *Trends Microbiol*. 2016;24(5):402-13.

16. Banerjee S, Schlaeppi K, van der Heijden MGA. Network analysis reveals functional redundancy and keystone taxa amongst bacterial and fungal communities during organic matter decomposition in an arable soil. *Soil Biol Biochem.* 2018;122:143-54.
17. Cleveland CC, Townsend AR, Schimel DS, Fisher H, Howarth RW, Hedin LO, et al. Global patterns of terrestrial biological nitrogen fixation in natural ecosystems. *Glob Biogeochem Cycles.* 1999;13(2):623-45.
18. Kesarcodi-Watson A, Kaspar H, Lategan MJ, Gibson L. Probiotics in aquaculture: The need, principles and mechanisms of action and screening processes. *Aquaculture.* 2008;274(1):1-14.
19. Rinke C, Schwientek P, Sczyrba A, Ivanova NN, Anderson IJ, Cheng JF, et al. Insights into the phylogeny and coding potential of microbial dark matter. *Nature.* 2013;499(7459):431-7.

Creative Commons (CC) License

This article is an open-access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY 4.0) license. This license permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.