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Biochar selectively enriches farm specific microbial species and supports niche differentiation in chicken manure across housing and feeding systems

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Abstract

Biochar is increasingly considered for treating chicken manure before residues are returned to farmland. It remains unclear whether biochar changes the whole manure-derived community or selectively enriches bacteria on its surface. This study investigated microbial community changes during 35 days of anaerobic incubation of manure from four broiler farms representing a 2 × 2 combination of feeding (probiotic or commercial feed) and housing (deep-litter or high-rise). Incubation was conducted with or without 10% (w/v) manure-derived biochar. Full-length Nanopore 16S rRNA gene sequencing characterised communities in fresh manure, post-incubation without biochar, and in biochar-liquid and biochar-solid fractions. Deep-litter manures were dominated by poultry lactobacilli (63.3–88.8%), whereas high-rise manures were rich in Proteobacteria (63.9–66.6%). During incubation, the liquid communities shifted towards *Escherichia coli* in three farms and *Alcaligenes faecalis* in the fourth. The mean between-farm Bray–Curtis dissimilarity fell from 0.86 to 0.61. Across all farms, the biochar-solid had lower relative abundance of *E. coli* and higher Shannon diversity than the adjacent liquid. One or two species were less abundant (≤3.5%) in that biochar-liquid but reached 14–51% on the biochar-solid. These dominant species varied among farms, and more than 98% of each biochar-solid community came from species detected in the same farm, including 18.5–58.8% from species present in its fresh manure. As each production system was represented by one farm, housing and feeding effects remain descriptive. Overall, biochar acted as a selective filter on farm-specific manure microbiomes. Recovered biochar therefore carries a farm-specific, manure-derived community and should be screened before land application.

Keywords: chicken manure, housing system, probiotic feeding, biochar colonisation, niche breadth, full-length Nanopore 16S rRNA sequencing

Introduction

The national chicken population in Malaysia increased from 285.3 million in 2021 to 326 million in 2024 [1]. Chicken manure (CM) is rich in nitrogen and organic matter [2], and improper disposal releases greenhouse gases and ammonia to the air, nutrients to surface waters and pathogens and odours to nearby communities [3, 4]. Most CM nitrogen is present as uric acid and urea, which microorganisms mineralise to ammonia [5–7]. High total ammonia nitrogen (TAN) inhibits methanogenic archaea and makes CM a difficult substrate for anaerobic treatment [8]. When methanogenesis is suppressed, volatile fatty acids (VFA) accumulate [9], and the removal of organic matter, measured as chemical oxygen demand (COD), depends more on hydrolytic, fermentative and non-methanogenic respiratory bacteria.

The microbial community entering treatment depends on upstream production practices. Malaysian broiler farms differ in housing, from high-rise houses in which excreta accumulate without bedding under continuous ventilation to deep-litter houses in which excreta mix with moist bedding, and in feeding, with or without probiotic supplementation. Litter bacterial communities differ between housing environments and between wet and dry litter [10, 11], and diet composition and probiotics alter excretion and the substrates available for degradation [12]. However, how housing and feeding shape manure communities, and whether these differences persist during treatment, remains poorly understood.

Biochar has been proposed as a stabilising amendment for CM digestion. Its porous structure and surface chemistry adsorb ammonium, buffer pH and provide surfaces for microbial attachment, reducing TAN and shortening the lag phase in CM digesters [8, 13]. Biochar produced at 400–700 °C also carries redox-active groups that allow it to act as an electron donor or acceptor for bacteria [14, 15], properties linked to microbial acclimation and direct interspecies electron transfer [16]. Biochar can selectively enrich functional microbes tightly attached to it [17], but most studies characterise the total community without separating biochar-associated microorganisms from the surrounding liquid. Whether biochar shifts the whole community or selectively recruits a subset onto its surface therefore remains unresolved, and the distinction matters when manures from different production systems supply different species pools.

Ecological theory predicts that a well-mixed liquid favours fast-growing copiotrophs, whereas a spatially structured surface allows slower, surface-adapted species to persist [18, 19]. These

predictions can be tested with 16S rRNA gene copy number as a proxy for potential growth rate [20, 21] and with niche breadth, which separates broadly distributed generalists from habitat specialists [22–24]. Because the organisms establishing in a new habitat depend on both selection and the species pool that reaches it [25], adding manure to a reactor is a community coalescence event [26] in which a common biochar filter could enrich different species from different manures. Identifying these species matters beyond the reactor, because manure-derived biochar is often returned to soil, where the charosphere can be enriched in antibiotic resistance genes [27], although CM biochar reduced some resistance genes during CM composting [28].

Resolving these patterns requires species-level identification. Short-read sequencing of partial 16S rRNA gene regions is biased by primer choice [29] and often cannot separate closely related species [30, 31], although species of the same genus can differ in pathogenicity and antibiotic resistance. Full-length Oxford Nanopore Technologies (ONT) 16S rRNA gene sequencing allows species-level assignment [32]; R10.4 chemistry reaches approximately 99% raw read accuracy [33], and Emu accounts for residual sequencing errors [31]. This approach has resolved species-level dynamics in the broiler caecum [34, 35] but has not yet been applied to manure treatment systems.

In this study, full length ONT 16S rRNA gene sequencing was selected because it provides long reads with lower capital cost, multiplexing and real-time data output. These features make species-level profiling feasible for manure microbiome studies without the infrastructure required by other long-read platforms. This study aims to (i) characterise the baseline microbiome taxonomic composition of fresh CM across different feeding regimes and housing systems; (ii) determine changes in microbial community composition over 35 days of anaerobic incubation alongside COD and VFA dynamics; and (iii) assess the effect of biochar amendment on microbial community composition and determine the specific species preferentially enriched in biochar relative to the surrounding liquid phase. We hypothesised that biochar would act as a selective ecological filter that preferentially enriches a subset of species associated with its surface rather than producing an equivalent shift across the bulk community. We further hypothesised that the identity of biochar-associated species would vary among manure sources because recruitment is constrained by the species pool available within each farm-derived community.

Methods

Research design and manure collection

Fresh CM was collected from four commercial broiler farms representing a 2 × 2 combination of feeding regime (probiotic and commercial feed) and housing system (high-rise and deep-litter). Broilers on the probiotic-fed farms received a commercial probiotic product (Otemchi®, Malaysia) throughout the production cycle whereas broilers on the commercial-fed farms received a conventional basal diet without probiotic supplementation. The manures were labelled as probiotic high-rise (PH), probiotic deep-litter (PD), commercial high-rise (CH) and commercial deep-litter (CD). On each farm, approximately 2 kg of CM was collected from 10 locations within the house and pooled into a single composite sample. Samples were transported on ice immediately after collection and stored at −20 °C.

Biochar preparation

The biochar was produced from CM collected at Universiti Putra Malaysia. The prewashed and oven-dried CM was pyrolysed in a muffle furnace under oxygen limited conditions at 430 °C for 30 min. The biochar was then ground, washed, passed through a 120-mesh sieve and re-pyrolysed at the same temperature to remove residual DNA [36]. The biochar was stored in a desiccator until incubation.

Anaerobic growth media preparation

Each thawed PH, PD, CH and CD manures was mixed with deionised water (1:5 w/v), stirred and gently boiled for 45 min. Suspension was collected after cooled, centrifuged at 4000 × g for 10 min, and sterilised through a 0.22 μm PES membrane. The residual CM was then extracted with 100 mL of ethanol (99%) with shaking for 16 h at ambient temperature [37]. The ethanol supernatant was removed by rotary evaporation at 50 °C and the residue was reconstituted to 200 mL with deionised water and sterilised through a 0.22μm PES membrane. Both water and ethanol extracts were stored in the dark at 4 °C and used within 1 week.

Mineral salt medium (MSM) was prepared according to Coleman Lab [38] with composition (g/L: K_2HPO_4 2.27, KH_2PO_4 0.95, $(NH_4)_2SO_4$ 0.67). Resazurin was added as a redox indicator (1 mg/L). The MSM containing resazurin solution was dispensed in 77.3 mL portions into 250 mL serum

bottles and boiled to reduce dissolved oxygen. The headspace was flushed with N_2 (99.999%), and sealed with butyl rubber stoppers and aluminium crimp caps before autoclaving at 121 °C for 15 min. Filter-sterilised trace element (0.2 mL) and vitamin (0.5 mL) solutions and L-cysteine·HCl (2 mL of 0.5 g/L) were then added, together with 5 mL of each manure extract as the only carbon sources and 10 mL of manure inoculum (1:10 in PBS), giving a working volume of 100 mL.

Anaerobic incubation

The incubations were designed as anaerobic microcosms in which the manure extracts were the only carbon source. The incubation period of 35 days. All incubations were conducted under strictly anaerobic conditions. Treatments without biochar (PH, PD, CH and CD) and with 10% (w/v) biochar (PHB, PDB, CHB and CDB) were prepared with one bottle per farm and treatment. The bottles were incubated at 37 °C and 120 rpm. Liquid samples (2 mL) were collected anaerobically on Days 0, 7, 14, 21, 28 and 35 for COD and VFA analyses.

COD and VFA Analyses

Samples were centrifuged at 10000 × g for 10 min and the supernatant was stored at –20 °C. COD analysis was conducted according to Hach Method 8000 using high range vials (20–1500 mg/L) and digested at 150 °C for 2 h in a DRB200 reactor (HACH Company, USA). COD was measured in technical triplicate with a DR3900 benchtop spectrophotometer (HACH Company, USA). VFA were determined by a method modified from Kareem et al. [39]. Samples were mixed 1:1 (v/v) with 24% metaphosphoric acid in 1.5 M 98% H_2SO_4 (R&M Chemical, Malaysia) and left overnight at room temperature. The mixture was then centrifuged at 10000 × g for 20 min at 4 °C. The supernatant was filtered through 0.45 µm and mixed 1:1 (v/v) with 10 mM 4-methyl-valeric (Sigma-Aldrich, USA) as internal standard. Acetic, propanoic and butyric acids were quantified by GC-FID (6890N, Agilent Technologies, USA) calibrated with external standards (Merck, Germany).

DNA Extraction and 16S Full Length Sequencing

DNA was extracted from each fresh manure and from every serum bottle on Day 35. In the biochar treatments, DNA from the biochar-solid and liquid phases was extracted separately. DNA extraction was conducted using the PrimeWay Soil DNA Extraction Kit (1st Base, Singapore)

according to the manufacturer's instructions. DNA quality and quantity were assessed using NanoDrop One spectrophotometer (ThermoFisher Scientific, USA).

For the Nanopore ONT sequencing, the V1-V9 hypervariable regions were amplified using 30 ng of DNA with LongAmp Hot Start Taq 2X Master Mix (2X) (New England Biolabs, USA), and the primers 27 F 5 ′ -AGAGTTTGATCMTGGCTCAG-3 ′ and 1,492 R 5 ′ -CGGTTACCTTGTTACGACTT-3′. Amplification was performed on a C1000 Touch Thermal Cycler (Bio-Rad, USA) using the program: 1 min denaturation at 95 °C, 25 cycles of 95°C 20 s, 55 °C 30 s, 65 °C 2 min and a final extension step of 2 min at 65 °C in 40 µL reactions. Library were prepared with the Rapid sequencing DNA-16S Barcoding Kit 24 V14 (SQK-16S114.24) kit (Oxford Nanopore Technologies Nanopore, UK). Libraries DNA concentration was assessed by Qubit dsDNA HS Assay Kit (Invitrogen, USA). Equimolar DNA pooled libraries were loaded onto R10.4.1 MinION flow cells and sequenced for 16 h following the manufacturer's instructions.

Bioinformatics Analysis

Reads were basecalled and demultiplexed with Dorado v2.1.0 [40] using the super-accurate model, assessed with NanoPlot v1.47.1 [41] and filtered with Chopper v0.11.0 [42] to retain reads with mean Q ≥ 10 and length 1,200–1,800 bp. Taxonomy was assigned with Emu v3.6.2 [31], which aligns reads with minimap2 [43] and estimates abundances by expectation–maximisation, using default parameters and its default database (rrnDB v5.6 [44] and NCBI 16S RefSeq [45]; 49,243 sequences). Species tables were aggregated to higher ranks with NCBI taxonomy. Functional groups were predicted with FAPROTAX v1.2.12 [46] by matching species-level taxonomy strings and expressed as a percentage of all reads.

Statistical analyses

Statistical analyses were conducted in R 4.6.1 with vegan 2.7-5. Emu counts were rarefied to the smallest library (89,266 reads; mean of 100 rarefactions), and species below 0.01% were removed; all percentages, diversity indices, dissimilarities and Table 1 are based on this table. Each production background was represented by a single house and each farm × treatment combination by a single library, so farm (n = 4) was the unit of replication, housing and feeding effects were treated descriptively, and all permutation tests were restricted within farms.

Community dissimilarity was quantified with Bray-Curtis and Aitchison distances and visualised by principal coordinates analysis (PCoA). The effect of sample type (fresh, post-incubation without biochar, biochar-liquid and biochar-solid) was tested by PERMANOVA with farm as the permutation block (9,999 permutations drawn from 331,776 possible), followed by blocked pairwise PERMANOVA between sample types. Multivariate dispersion was checked with betadisper. Observed richness, Shannon, inverse Simpson and Pielou evenness were compared among sample types with Friedman tests blocked by farm using exact *p*-values obtained by 331,776 within-farm permutations. Chao1 was not calculated because Emu does not output singletons.

Bray–Curtis and Aitchison dissimilarities were visualised by principal coordinates analysis (PCoA). The effect of sample type (fresh, without biochar, biochar-liquid and biochar-solid) was tested by PERMANOVA with farm as the permutation block (9,999 permutations), followed by blocked pairwise tests, and dispersion was checked with betadisper. Observed richness, Shannon, inverse Simpson and Pielou evenness were compared with Friedman tests blocked by farm using exact within-farm permutation *p*-values. Between-farm divergence was summarised as the mean Bray–Curtis dissimilarity of the six farm pairs for taxonomic and predicted functional profiles.

The biochar-solid and liquid of each bottle were compared as pairs. For the 37 species exceeding 1% in either fraction in any farm, $\log_2$ solid-to-liquid ratios were calculated per farm and the number of farms with more than twofold enrichment or depletion was recorded. ANCOM-BC2 v2.14.0 [47] was applied to unrarefied genus counts. Because the smallest attainable exact *p*-value with four farms is 0.0625 (one-sided sign test), effect sizes and consistency of direction are reported rather than significance. Dominant colonisers (≥ 5% on the solid and ≥ 2-fold above the liquid) were tracked across all samples (Table 1), and the reads on each solid were assigned, in order of priority, to species detected in the fresh manure of the same farm, only in liquids of the same farm, only in other farms, or only on the solid. Solid–liquid membership differences were partitioned into replacement and nestedness components of Sørensen dissimilarity [48]. Robustness was checked by repeating the PERMANOVA without solid samples, correcting for 16S rRNA gene copy number and varying the detection threshold (0.05% and 0.1%) and pseudocount (10^{-3}). Enriched and other abundant species were compared for oxygen requirement, motility, Gram stain, cell shape and sporulation (Fisher's exact test).

Trait-based analysis and Levins' niche breadth

Species were assigned 16S rRNA gene copy number from the condensed species table of the bacterial and archaeal phenotypic trait database [49]. The mean for the genus, family, order or class was used where no species value existed. Functional groups were predicted with FAPROTAX v 1.2.12 [46] by matching taxonomy strings that included the species name and expressed as a percentage of all reads.

Niche breadth was calculated for the 90 species exceeding 1% relative abundance in at least one sample, using a single rarefied draw. Because each farm and sample type were represented by one library, niche breadth was used descriptively. For each species, p_i is the proportion of its reads found in sample (or sample type). Levins' niche breadth [50] across all 16 samples was calculated with the spaa package v 0.2.5 [51] as

$$B = \frac{1}{\sum p_i^2} \quad (1)$$

and standardised as

$$B_A = \frac{B - 1}{n - 1} \quad (2)$$

with n = 16, B_A ranges from 0 (a species confined to one sample) to 1 (a species equally distributed across all samples). Levins' B_n across the R = 4 sample types were calculated with the MicroNiche package v 1.0.0 [23, 52], both across farms and within each farm. The B_n was calculated as

$$B_n = \frac{\frac{1}{R}}{\sum p_i^2} \quad (3)$$

which ranges from 1/R (a species confined to one sample type) to 1 (a species equally distributed across all sample types). Species with B_n significantly below the null expectation for a random distribution (Benjamini–Hochberg adjusted $p < 0.05$; $B_n \leq 0.57$) were classified as narrow niche taxa whereas $B_n > 0.57$ as broad niche taxa. The classification was checked by repeated rarefactions, by resampling whole farms and omitting each farm in turn.

Results

Sequencing depth and overall community structure

After quality and length filtering, the 16 libraries contained 3,975,460 reads (5.81 Gb; 248,466 ± 193,503 reads per library, range 89,401–603,531), with median read lengths of 1,385–1,485 bp and median Q scores of 23.2–25.5. Community composition was associated with sample type within farms (blocked PERMANOVA, Bray-Curtis $R^2 = 0.267$, $F = 1.46$, $p = 0.0013$; Aitchison $R^2 = 0.230$, $p \leq 0.0001$). In the ordination, the fresh manures occupied separate regions, whereas all post-incubated samples moved towards a common region (**Fig. 1a**). Within each farm, the fresh and post-incubation communities were highly different (Bray-Curtis 0.69–0.96), as shown in **Fig. 1b**. Shannon diversity fell from 2.09–4.21 in fresh manure to 1.51-2.44 after incubation without biochar and 1.23–1.98 in biochar-liquid. Alpha diversity differed among sample types (Friedman test blocked by farm: Shannon $\chi^2 = 9.9$, exact $p = 0.006$; with the same result for inverse Simpson and Pielou evenness; observed richness $\chi^2 = 6.9$, exact $p = 0.068$).

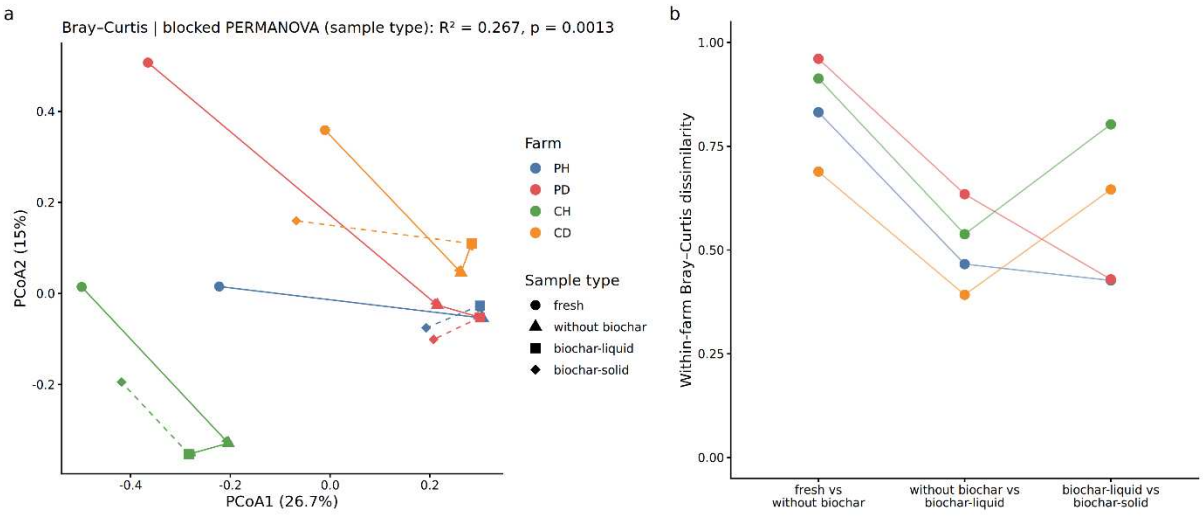


Fig. 1 (a) PCoA of Bray-Curtis dissimilarities for all 16 libraries. Colour, farm (PH is probiotic high-rise, PD is probiotic deep-litter, CH is commercial high-rise and CD is commercial deep-litter); shape, sample type. Solid lines connect the fresh manure to without biochar and then to biochar-liquid whereas dashed lines connect each biochar-solid to the biochar-liquid in the same treatment. Blocked PERMANOVA for sample type: $R^2 = 0.267$, $p = 0.0013$. (b) Within-farm Bray-Curtis dissimilarity for three contrasts. One library per farm and sample type; the farm (n = 4) is the unit of replication.

Fresh manure communities

Across the Emu libraries, the two deep-litter manures were the most similar pair of fresh samples (Bray-Curtis 0.65) as shown in **Fig. 1a**, whereas all other pairs were more dissimilar (0.82–0.95).

Deep-litter manure was dominated by Firmicutes (PD 94.7%, CD 66.9%), mainly *Lactobacillus* (PD 88.8%, CD 63.3%) in **Fig. 2** and **Fig. 3** and had lower diversity (Shannon 2.09–2.21; Pielou 0.47–0.49) than high-rise manure (Shannon 3.85–4.21; Pielou 0.70–0.74).

High-rise manure was dominated by Proteobacteria (PH 66.6%, CH 63.9%), and the two high-rise manures differed from each other (Bray-Curtis 0.89) as much as from deep-litter manure. PH was dominated by *Proteus* (12.6%; *P. mirabilis* 11.3%), *Escherichia* (11.3%), *Providencia* (11.1%; *P. rettgeri* 9.6%) and *Acinetobacter* (10.0%). CH had the most even community (Pielou 0.74) and was characterised by *Luteimonas* (18.2%; *L. granuli* 8.8%), *Sphingobacterium* (10.7%), *Oceanimonas smirnovii* (5.5%), *Idiomarina indica* (5.2%), *Alcaligenes faecalis* (4.5%) and *Pusillimonas noertemannii* (4.0%).

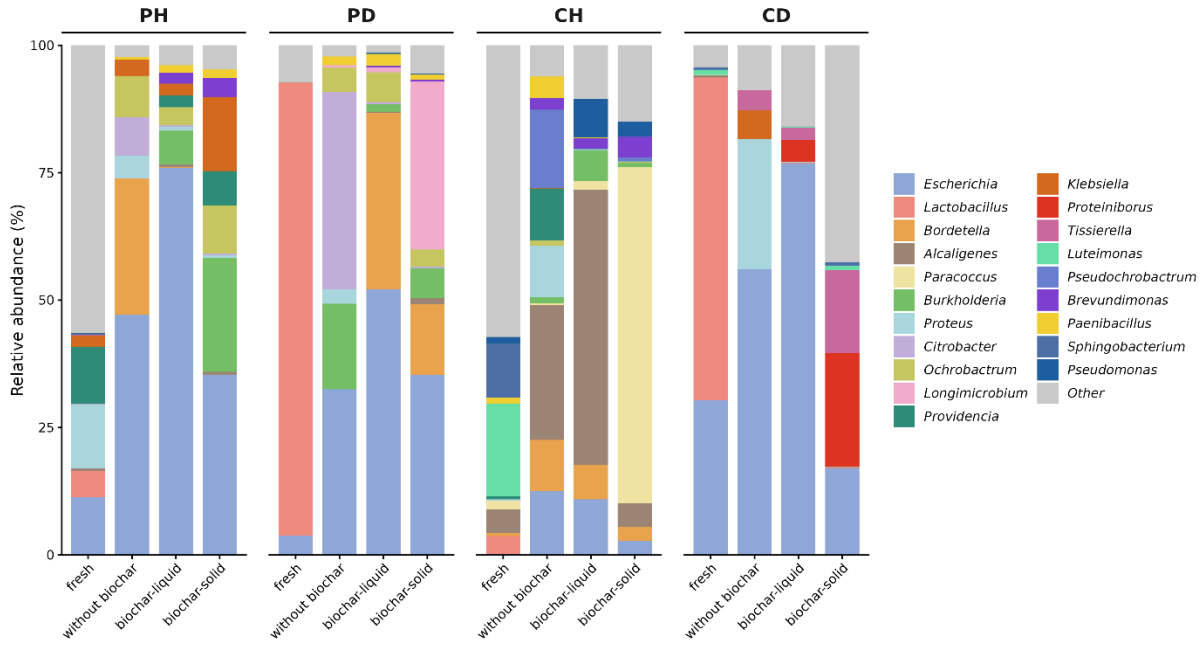
Housing systems had a larger effect on lactobacilli than feeding regime. On the probiotic farms, *Lactobacillus* made up 88.8% of deep-litter manure but only 5.1% of high-rise manure whereas 63.3% and 3.5% in commercial farms respectively. The same poultry-associated species were present in high-rise manure, but at low abundance (*L. aviarius* 2.6% in PH and 2.2% in CH). *E. coli* showed no consistent relation with feeding. In deep litter, the PD (3.9%) carried less *E. coli* than the CD (30.4%), whereas in high-rise manure the PH (11.3%) carried more than the CH (0.2%).

Post-incubation liquid communities

After incubation without biochar, Proteobacteria made up 88.8–97.2% of every community, and lactobacilli fell below 0.3% (**Fig. 2** and **Fig. 3**). *E. coli* became the most abundant species in PH (47.2%) and CD (56.1%) and co-dominated PD (32.5%) with *Citrobacter sedlakii* (38.6%). Secondary species differed between farms: *Bordetella trematum* in PH (26.7%), *P. mirabilis* in CD (25.5%), and in CH *A. faecalis* (26.4%), *Pseudochrobactrum asaccharolyticum* (15.3%), *E. coli* (12.5%), *P. rettgeri* (10.1%), *P. mirabilis* (10.0%) and *B. trematum* (10.0%). CD was the only system in which strictly anaerobic, proteolytic Firmicutes were detected in the liquid, most clearly in the biochar liquid (*Gallicola barnesae* 5.2%, *Proteiniborus ethanoligenes* 3.5%, *Peptoniphilus ivorii* 2.4%, *Tissierella creatinini* 2.4%).

The biochar-liquid communities retained the dominant species of the corresponding treatment without biochar. *E. coli* in PH (75.9%), PD (52.1%) and CD (76.8%), *B. trematum* in PD (34.3%)

297 and *A. faecalis* in CH (54.0%). In every farm, the biochar liquid was less diverse than the liquid
298 without biochar (Shannon –0.24 to –0.46).



299

300 **Fig. 2** Relative abundance of the 20 most abundant genera in fresh manure, after 35 days of incubation without biochar,
301 and in the biochar-liquid and biochar-solid fractions of the biochar-amended treatments (Emu, NCBI/rrnDB database;
302 rarefied libraries). Remaining genera are grouped as Other, which is largest in the diverse high-rise fresh manures.

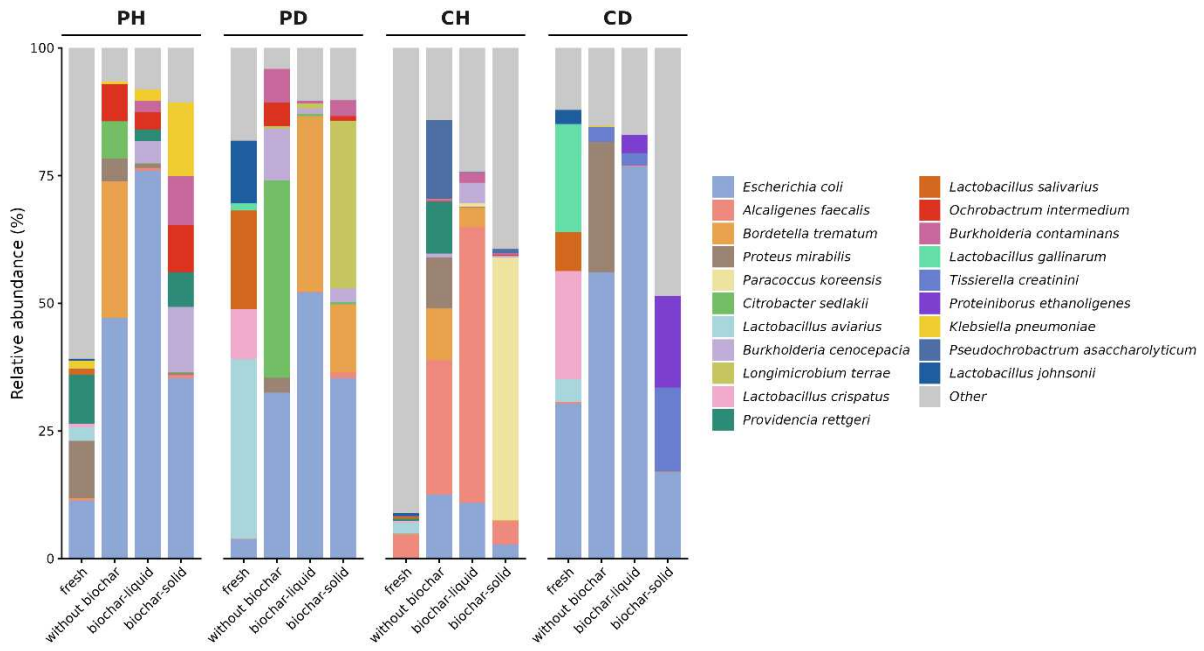
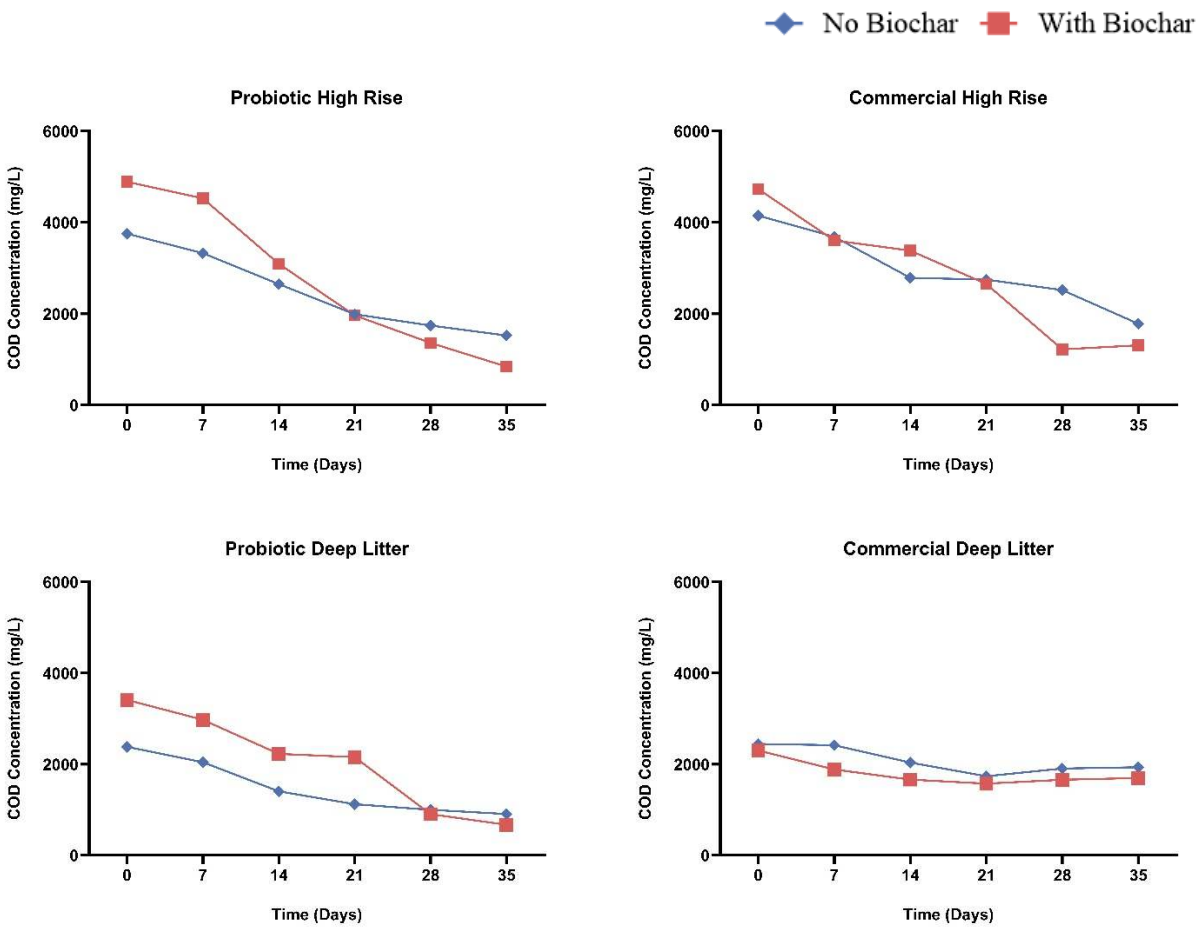


Fig. 3 Relative abundance of the 20 most abundant species in fresh manure, after 35 days of incubation without biochar, and in the biochar-liquid and biochar-solid fractions of the biochar-amended treatments (Emu, NCBI/rrnDB database; rarefied libraries). Remaining species are grouped as Other, which is largest in the diverse high-rise fresh manures.

COD and VFA dynamics during incubation

COD declined steadily over 35 days in PH, PD and CH, falling by 57 to 62% without biochar and continuing to decline after VFA had dropped below detection at Day 21 (**Fig. 4** and **Fig. 5**). However, COD of CD sample fell by only 21%, reached a minimum at Day 21 and then increased slightly to a plateau. Biochar-amended treatments of PH, PD and CH started with higher COD than those without biochar but ended lower, with most of the additional decline in PD and CH occurring between Days 21 and 28. In CD, biochar had little effect which fell by 21% in without biochar and 26% with biochar. Because biochar itself contributed soluble COD at Day 0, part of the greater decline in the biochar-amended treatment may reflect removal of biochar-derived organics rather than enhanced degradation of manure organic matter.



317

318 **Fig. 4** Chemical oxygen demand (COD) in the liquid phase over 35 days of anaerobic incubation of manure from
319 probiotic high-rise (PH), probiotic deep-litter (PD), commercial high-rise (CH) and commercial deep-litter (CD)
320 houses, without (blue diamonds) and with 10% (w/v) biochar (red squares). Panels are arranged by housing (columns)
321 and feed (rows) for direct comparison

322 Acetic acid was the highest VFA in all treatments at every timepoint and accounted for 79 to 100%
323 of total VFA at Day 7. In PH, PHB, PD, PDB, CH and CHB, total VFA followed the same pattern
324 where concentration peaked at Day 7 and declined sharply by Day 14 and fell below the limit of
325 detection by Day 21. However, acetic, propanoic and butyric acid in CD and CDB followed distinct
326 pattern where increased gradually to a plateau and persisted to Day 35. Biochar amendment
327 reduced VFA concentrations in PDB and CHB at Day 7.

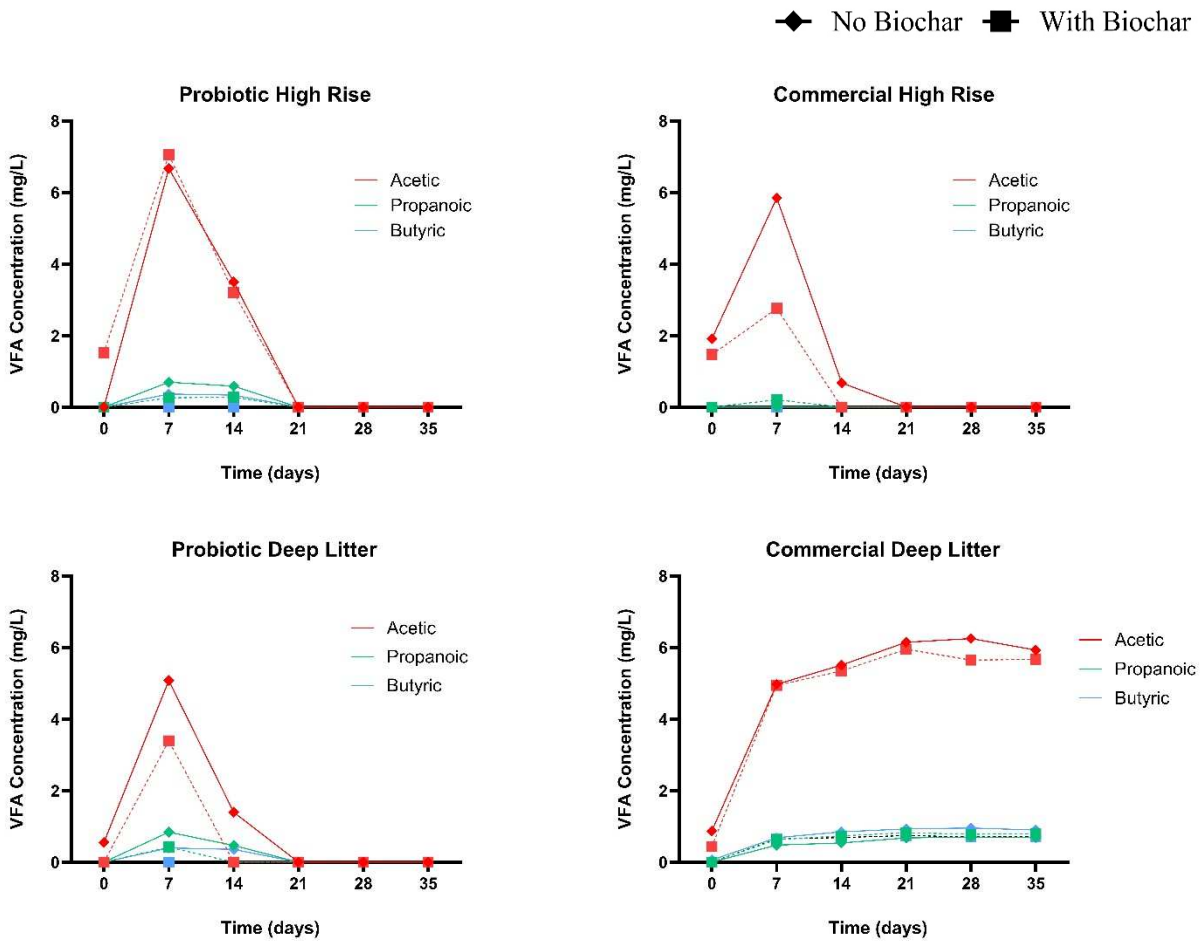


Fig. 5 Acetic, propanoic and butyric acid in the liquid phase without (solid lines, diamonds) and with biochar (dashed lines, squares). Panels are arranged by housing (columns) and feed (rows) for direct comparison

Biochar-solid compared with the biochar-liquid of the same treatment

The biochar-solid did not differ from the adjacent liquid as a whole community (Bray-Curtis R^2 = 0.135, exact $p = 0.25$), but three differences between the biochar-solid and the adjacent liquid were consistent across all four farms (**Fig. 2**, **Fig. 3** and **Fig. 6**). Interestingly, *E. coli* was less abundant on the solid (PH 35.3%, PD 35.4%, CH 2.7% and CD 17.0%; $\log_2$ ratio –0.56 to –2.18; 4 of 4 farms, exact one-sided $p = 0.0625$). *E. coli* was the only one of 37 species exceeding 1% in either fraction that changed in the same direction in every farm. In addition, the solid had higher Shannon diversity than its liquid in all four farms (+0.17 to +1.77), and higher evenness in three farms (Pielou +0.11 to +0.33), with equal evenness in CH (+0.006). Besides, each solid was dominated by one or two species that were minor in the adjacent liquid like *K. pneumoniae* in PH (14.4% vs. 2.2%; 6.5-fold), *Longimicrobium terrae* in PD (32.9% vs. 1.0%; 33-fold), *Paracoccus koreensis*

342 in CH (51.4% vs. 0.7%; 76-fold), and *P. ethanoligenes* (17.9% vs. 3.5%; 5.1-fold) and *T. creatinini*
343 (16.4% vs. 2.4%; 6.9-fold) in CD. As expected with four farms, ANCOM-BC2 (farm as a fixed
344 block, Holm correction) identified no genus that differed significantly among sample types.

345 The identity of the enriched species differed between farms. Of the 37 abundant species, 18 were
346 enriched more than twofold on the biochar on only one farm, 8 in two, 1 in three (*Ochrobactrum*
347 *intermedium*) and none in all four (**Fig. 6**). The CH biochar was dominated by *Paracoccus* (66.0%;
348 *P. koreensis* 51.4%, *P. solventivorans* 14.0%), the CD biochar by Firmicutes (64.5%;
349 *Proteiniborus* 22.3%, *Tissierella* 16.4%) with Bacteroidetes (15.3%), the PD biochar by *L. terrae*
350 (Gemmatimonadetes, 32.9%) with *E. coli* (35.4%) and *B. trematum* (13.2%), and the PH biochar
351 by *Burkholderia* (22.4%; *B. cenocepacia* 12.7%, *B. contaminans* 9.6%), *K. pneumoniae* (14.4%)
352 and *O. intermedium* (9.3%).

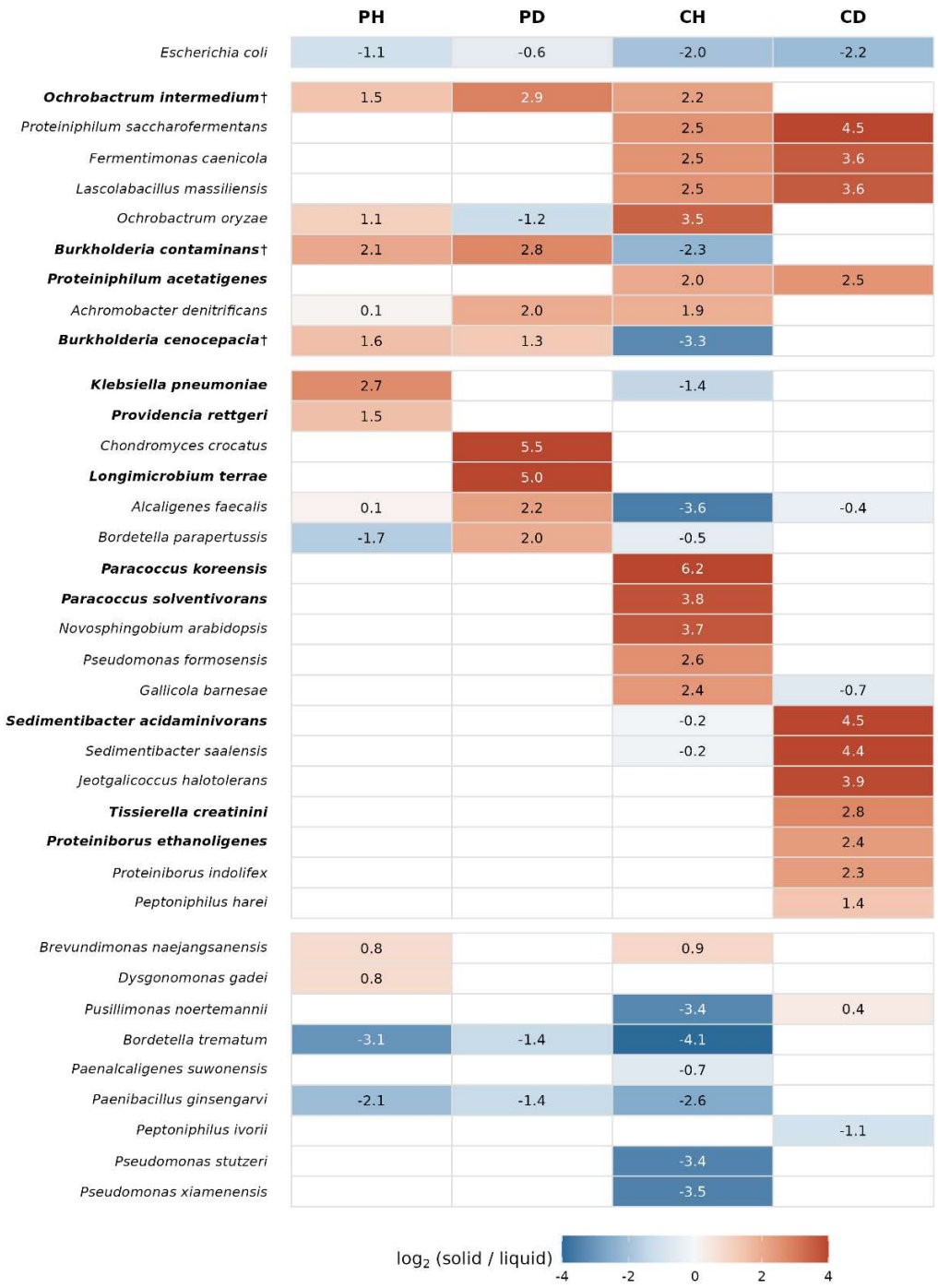


Fig. 6 Relative enrichment in the biochar-solid fraction compared with the biochar-liquid of the same bottle, $\log_2[(\text{solid} + 10^{-4})/(\text{liquid} + 10^{-4})]$, for the 37 species exceeding 1% in either fraction in any farm (rarefied table). Values are raw relative-abundance ratios; where *E. coli* was depleted on the solid (PH and CD), they are inflated for all other taxa. Blank cells mark species absent from both fractions of that farm. Rows are grouped from top to bottom into species lower on the biochar in every farm (*E. coli* only), enriched more than twofold in two or more farms, enriched more than twofold in one farm only and not enriched more than twofold in any farm. Colours are capped at ± 4; printed values are exact. Bold names are the dominant biochar colonisers of **Table 1** (≥ 5% on the solid and more than twofold above the liquid); †, possible reagent- or medium-derived taxa (**Table 1**)

Origin of the biochar colonisers

Table 1 traces each dominant coloniser across the samples of its farm. *K. pneumoniae*, *P. rettgeri*, *T. creatinini* and *Proteiniphilum acetatigenes* were detected in the fresh manure of the same farm. However, *B. cenocepacia*, *B. contaminans* and *O. intermedium* were not detected in fresh manure from any farm but appeared after incubation in three farms (PH, PD and CH), including bottles without biochar (10.1%, 6.6% and 7.2%, respectively) and were absent from all CD samples. These species came from rare manure populations or from reagents or medium ([a], Table 1) cannot be determined without an extraction blank.

At the community level, 98.3 to 99.7% of the reads on each biochar-solid belonged to species detected elsewhere in the same farm: 18.5 to 58.8% to species present in its fresh manure and 40.9 to 80.1% to species detected only in that farm's liquids. The biochar-solid and the biochar-liquid of the same bottle shared most species (Sørensen dissimilarity 0.25–0.31), with replacement (0.12–0.24) and nestedness (0.01–0.14) components. Abundance-based dissimilarity between the two fractions was higher (Bray-Curtis 0.43-0.80). Between farms, the liquids converged during incubation (mean Bray-Curtis between farm pairs 0.86 in fresh manure, 0.66 without biochar and 0.61 in the biochar liquid), whereas the biochar solids remained as distinct from one another as the fresh manures (0.84), although part of this difference reflects the lower share of the liquid-dominant species on the solids.

Table 1 Taxonomic detection patterns of dominant biochar-associated taxa (≥ 5% on the solid and ≥ 2-fold above the adjacent liquid): relative abundance (%) across the samples of the same farm

Farm	Species	Fresh	Without Biochar	Biochar-liquid	Biochar-solid	Farms detected
PH	*Burkholderia cenocepacia* [a]	0.00	0.00	4.33	12.7	PH, PD, CH
PH	*Burkholderia contaminans* [a]	0.00	0.00	2.24	9.6	PH, PD, CH
PH	*Klebsiella pneumoniae*	1.51	0.54	2.23	14.4	PH, CH, CD
PH	*Ochrobactrum intermedium* [a]	0.00	7.23	3.37	9.3	PH, PD, CH
PH	*Providencia rettgeri*	9.63	0.00	2.32	6.7	PH, CH
PD	*Longimicrobium terrae*	0.00	0.45	1.01	32.9	PD
CH	*Paracoccus koreensis* [b]	0.00	0.00	0.68	51.4	CH
CH	*Paracoccus solventivorans*	0.00	0.49	1.00	14.0	CH
CD	*Proteiniborus ethanoligenes* [b]	0.00	0.00	3.48	17.9	CD
CD	*Proteiniphilum acetatigenes*	0.03	0.05	0.89	5.1	CH, CD
CD	*Sedimentibacter acidaminivorans* [b]	0.00	0.00	0.26	5.9	CH, CD

CD	*Tissierella creatinini*	0.02	2.97	2.36	16.4	PH, CD

0.00 = not detected after rarefaction and the 0.01% filter; farms detected counted at ≥ 0.015% in any sample of that farm. [a] Possible reagent- or medium-derived taxa: genera listed as reagent contaminants by Salter et al. [53] that were absent from all fresh manures but present in bottles without biochar in three farms; interpreted with caution because no extraction blank was sequenced. [b] Detected only in biochar-amended treatments; their presence in rare manure populations cannot be distinguished from introduction through the biochar or medium. *Paracoccus* is also listed by Salter et al. [53] but was confined to one farm and dominated a high-biomass sample, which argues against a reagent origin.

Predicted functional profiles shifted between fresh and post-incubation communities

In **Fig. 7** and **Fig. 8**, FAPROTAX assigned 75.4–97.6% of reads to at least one functional group in most samples, but only 44.7% in CH fresh manure, 63.8% on the PD biochar-solid and 33.0% on the CD biochar-solid, due to *L. terrae* and *Proteiniborus* have no annotation. In the biochar-liquid, *E. coli* supplied 92.1 to 98.6% of the fermentation activity in all four farms and 99.5 to 99.9% of the predicted nitrate-respiration activity in PH, PD and CD, but only 58.7% in CH, where it made up 10.9% of reads. The CH biochar-solid had the highest proportions of denitrification (71.9%) and methylotrophy (66.0%) and a high proportion of nitrate respiration (75.7%), mainly because of the presence of *P. koreensis*. Compared with the adjacent liquid, fermentation, fumarate respiration and pathogen-annotated species were lower on the solid in all four farms ($\log_2$ –0.3 to –2.4), whereas denitrification, methylotrophy, ureolysis and aromatic compound degradation increased only in some farms (**Fig. 8c**).

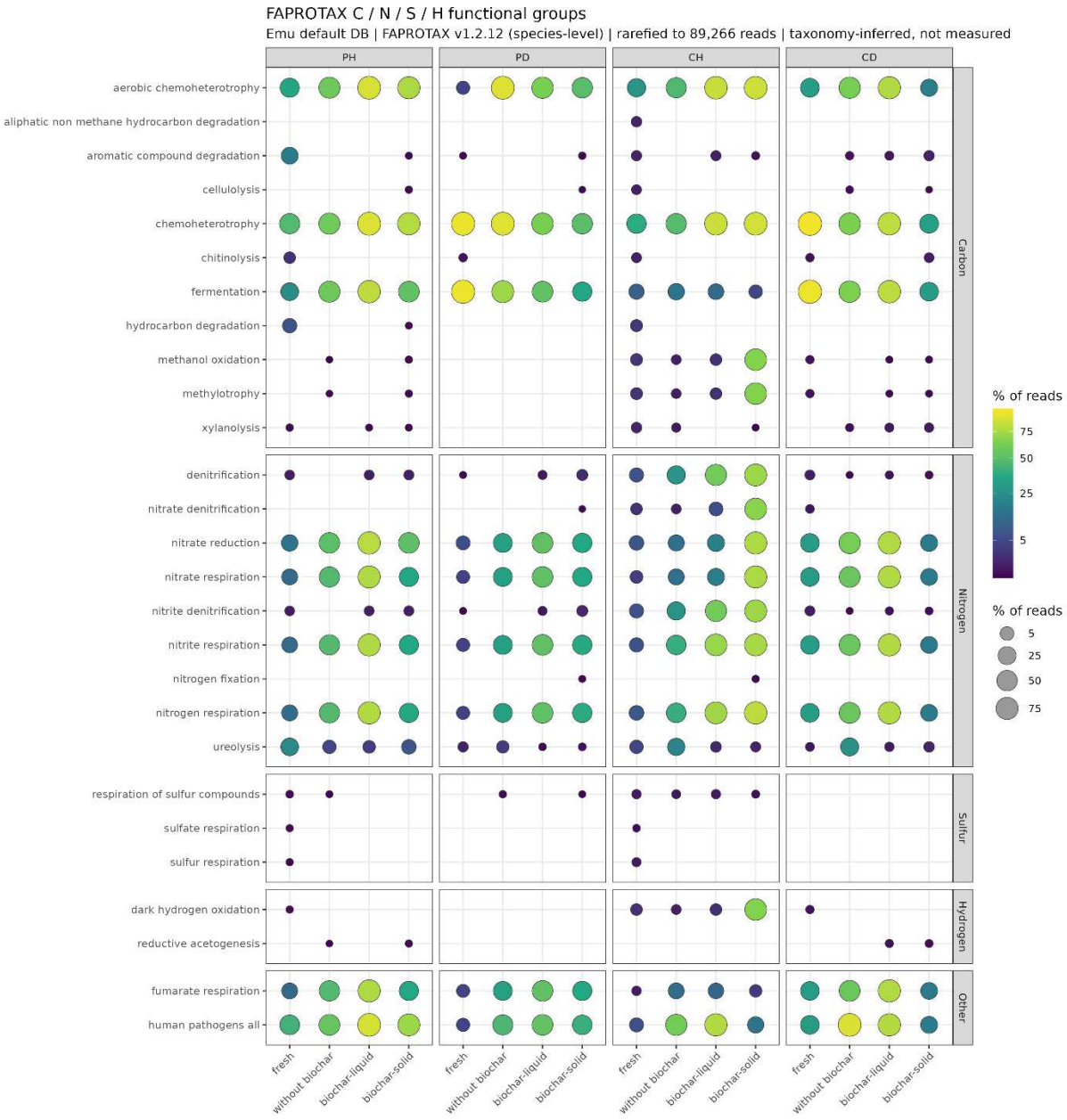


Fig. 7 Predicted functional groups (FAPROTAX v 1.2.12, species-level) as % of reads in the rarefied table. Colour and circle size are on a square-root scale. Functions are inferred from taxonomy and were not measured

Growth strategy and niche breadth

Community-weighted 16S rRNA gene copy number, a genomic proxy for maximal growth rate [20, 21], covered ≥ 99% of reads in every sample except PD biochar-solid (67%). It increased during incubation without biochar in three farms, PD (+1.37), CH (+0.85) and CD (+ 1.08), and did not change in PH (**Fig. 8a**). The biochar-solid had a lower copy number than biochar-liquid in

all four farms (–0.12 to –1.58; exact one-sided $p = 0.0625$). This difference disappeared when *E. coli* (7.1 copies) was excluded, so it reflects the depletion of *E. coli* alone.

Overall, 80 of the 90 abundant species were classed as narrow niche (B_n 0.25–0.57, adjusted $p < 0.05$) and 10 as broad niche. *E. coli* was the most abundant broad niche species ($B_A = 0.60$; $B_n = 0.79$), whereas the dominant biochar colonisers had narrow niches (B_A 0.002–0.045; B_n 0.26–0.42) in **Fig. 8b**. The colonisers were largely farm specific. Of the 21 narrow niche species most abundant on the biochar solid, only six showed this pattern in more than one farm.

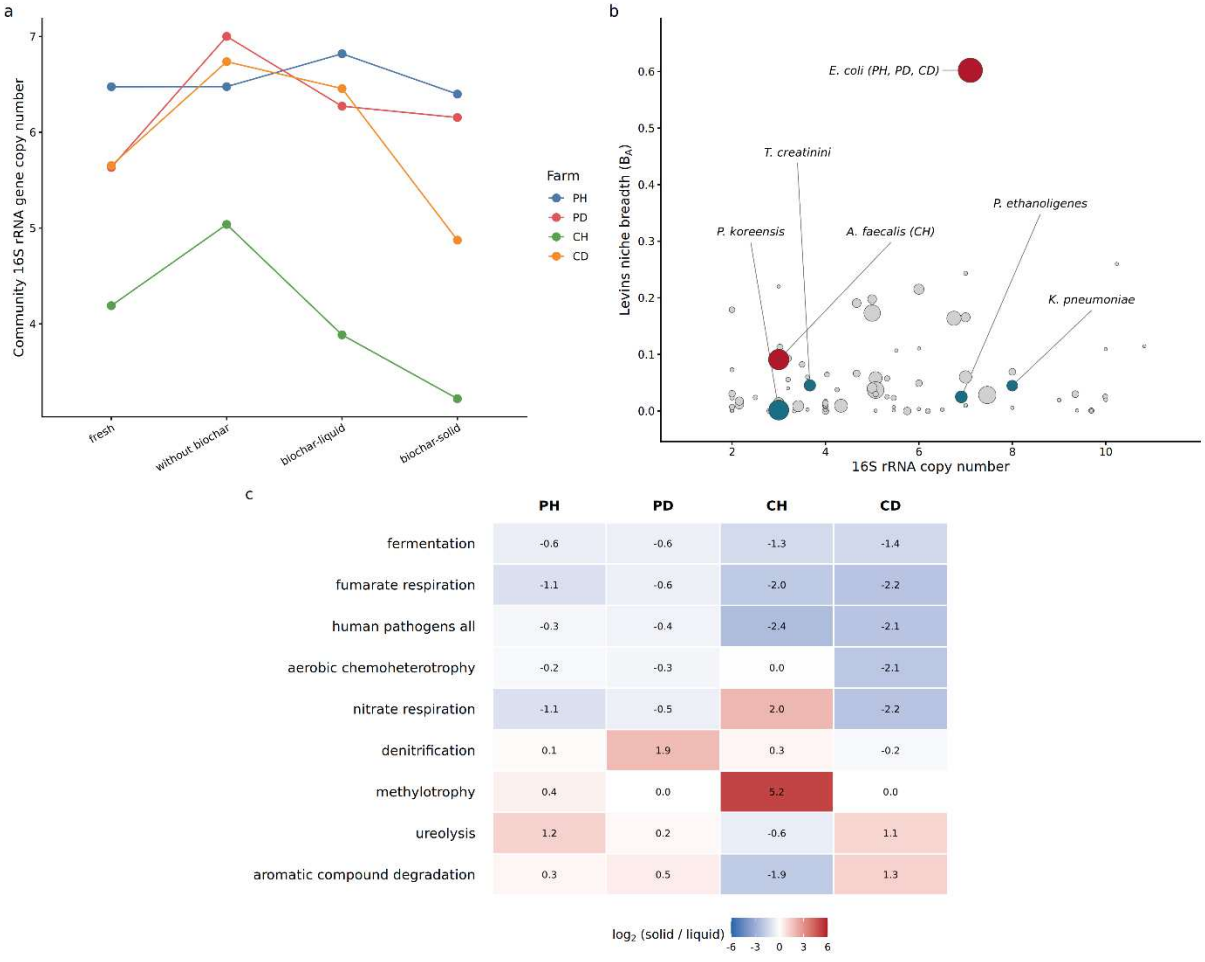


Fig. 8 Growth strategy, niche breadth and predicted function. (a) Community-weighted 16S rRNA gene copy number per sample [49]; lines join the samples of each farm. (b) Levins' standardised niche breadth (BA) across the 16 samples against species copy number for species exceeding 1% in any sample (point size, maximum abundance); dominant species of the liquids in red (E. coli in PH, PD and CD; A. faecalis in CH), dominant biochar colonisers in blue and other species in grey. Species without copy-number data at class level or below, including L. terrae, are not shown. (c) $\log_2$ ratio of FAPROTAX functional-group abundance on the biochar solid to that in the biochar liquid of the same bottle (pseudocount 0.1%)

Discussion

Deep-litter manure retained more of the gut-derived lactobacilli than high-rise manure

Housing system associated with larger differences between the fresh manures than feeding regime. The two deep-litter manures were the most similar pair and shared a low-diversity consortium of poultry lactobacilli. This is consistent with a largely gut-derived community and with earlier reports that broiler litter is dominated by low G+C Gram-positive bacteria [11]. The two high-rise manures were more diverse and differed from each other as much as from deep-litter. PH was rich in the urease-positive Morganellaceae *Proteus* and *Providencia*, whereas CH carried halo- and alkali-tolerant genera like *Idiomarina*, *Oceanimonas*, *Luteimonas* and *Alcaligenes*. This is because mineralisation of uric acid and urea to ammonia depends on moisture, pH and time [6, 7], these two communities may represent different stages of ex-host succession, ureolysis-dominated in PH and a later saline–alkaline stage in CH. These results suggest that high-rise housing allows excreted communities to diverge along farm-specific paths, whereas deep-litter retains a gut-derived signature.

The same *Lactobacillus* were detected at low abundance in high-rise manure (*L. aviarius* 2.2–2.6%), which suggests that they were lost after excretion rather than absent from the gut. High-rise manure accumulates and ages under the slats for the whole cycle, and *Lactobacillus* lose dominance once outside the host [54]. Due to the probiotic effects on caecal composition can fade within weeks as the community matures, even under continuous dosing [55], repeated exposure through the litter may help sustain probiotic signals. In deep-litter, the probiotic farm carried more lactobacilli and about eightfold less *E. coli* than the commercial farm.

Full length Nanopore species-level resolution also reveals possible ecological roles for the dominant species in each manure. The deep-litter *Lactobacillus* taxa (*L. aviarius*, *L. salivarius*, *L. johnsonii*, *L. crispatus*, *L. reuteri* and *L. gallinarum*) are associated with the chicken small intestine [56], so their dominance indicates that deep-litter manure remained close to its gut origin. In contrast, some of the dominant high-rise species are associated with the environment or with manure transformed outside the host. *Proteus mirabilis* and *Providencia rettgeri* are urease-positive, and urea hydrolysis by *P. mirabilis* releases ammonia and raises pH [57], whereas *Alcaligenes faecalis* can remove ammonium by heterotrophic nitrification and aerobic denitrification [58]. The high-rise manures therefore combined taxa that may generate ammonia

with taxa that may transform it. High-rise manure therefore brings a larger and functionally different species pool into treatment, including opportunistic pathogens such as *P. mirabilis* [57]. Because these roles were inferred from taxonomy rather than measured, they should be confirmed with ammonia, urease activity and metagenomic data.

Incubation and the fate of organic carbon

Anaerobic incubation removed most between farm differences in the liquid, which became dominated by *E. coli* in three farms and by *A. faecalis* in CH. VFA remained at low concentrations throughout (Figure 5) and therefore accounted for only a small fraction of COD. Thus, the COD dynamics mainly reflect other soluble organic compounds. The continued decline of COD in PH, PD and CH after VFA had disappeared may reflect assimilation into biomass removed by centrifugation, loss of H_2 and CO_2 to the headspace, respiration coupled to electron acceptors in the extracts, or methanogenesis by archaea that our primers could not detect. FAPROTAX attributed nitrate respiration mainly to *E. coli* (Figure 7), but water extracts of chicken manure contain little nitrate [59], so the predictions unable to distinguish between these explanations. In CD, the persistence of VFA and of a proteolytic Firmicute guild suggests continued amino acid fermentation, although at these concentrations VFA alone cannot explain the COD plateau.

Biochar as a selective niche filter with farm-specific colonisers

The distribution of *E. coli* suggests that the liquid and the biochar surface acted as distinct niches favouring different growth strategies. Fast-growing copiotrophs are expected to dominate well-mixed liquids where substrates are uniformly available [19], whereas spatially structured surfaces with adsorbed substrates allow slower, surface-adapted species to persist [18]. Although *E. coli* may form biofilms on biochar [60], its lower share on the solid indicates that other species preferentially occupied the surface. Relative abundances cannot show whether these species displaced *E. coli* or simply established first [61], which would require absolute quantification over time.

In contrast, the identity of the enriched species varied by farm. Each dominant coloniser was absent or minor in the other farms, and 18 of the 37 abundant species were enriched in only one farm. This suggests that a common selective filter acted on species pools that differed between inocula, consistent with environmental filtering combined with historical contingency [25]. Similarly, the

bacteria colonising swine-manure biochar in soil depended on both pyrolysis temperature and soil type [36]. Priority effects or drift within a single bottle may also have determined which species dominated [62], so the consistent direction of selectivity across farms is better supported than the identity of any coloniser. Source tracking indicated an inoculum origin, as more than 98% of reads on each solid belonged to species detected in the same farm and less than 1% to species found only in other farms. A taxon carried in by the single biochar batch would be expected in all four farms, yet *L. terrae* and *P. solventivorans* were confined to their own farm, and the *K. pneumoniae* and *T. creatinini* were present in fresh manure. The biochar-solid and biochar-liquid shared most species but differed in their abundances, so the biochar mainly reweighted the local species pool. While the biochar-liquids converged between farms, the biochar surfaces remained as distinct as the fresh manures and retained a farm-specific signature.

Three observations constrain how the colonisers came to dominate the biochar-solid. First, enrichment was selective rather than passive. Despite the unwashed pellet, 46-81% of reads on each solid belonged to species at least twofold more abundant there than in the liquid, and solid and liquid abundances were only moderately correlated (Spearman ρ = 0.51-0.61). Second, absent of single trait distinguished the 27 enriched species from the other 10, as both groups included aerobes and strict anaerobes, motile and non-motile taxa, and Gram-positive and Gram-negative taxa, with similar median 16S rRNA gene copy numbers (3.4 and 4.0). Third, predicted functions converged between farms in the liquids (mean Bray-Curtis 0.28-0.29) but not on the solids (0.49), where each surface carried farm-specific functions such as denitrification and methylotrophy on CH and proteolytic fermentation on CD. These patterns are consistent with habitat filtering of farm-specific species pools rather than a universal biochar-colonising trait.

Growth strategy and niche breadth of the colonisers

E. coli was the dominant species of the liquids, has a broader niche than the dominant biochar colonisers. A predominance of habitat specialists over generalists is a common pattern in microbial communities [22], and surfaces under strong selection show the same tendency. Visually distinct biofilms on red sandstone had narrower Levins' niche breadth and more habitat specialists than control surfaces [24]. In contrast, river biofilms, which are continuously seeded from a connected network, are dominated by high-occupancy generalists [23]. In the sealed bottles, each biochar could draw only on its own inoculum, which may have favoured local and narrow niche colonisers.

This is because most colonisers were detected in only one or two farms and their narrow niches describe this experiment rather than a general preference of these species for biochar.

Genomic traits indicate that the surface admitted both fast and slow growers. Community 16S rRNA gene copy number increased during incubation in three farms, as expected when a nutrient pulse favours copiotrophs [20, 21]. Its lower value on the biochar-solid than in the biochar-liquid reflected the depletion of *E. coli* alone. Some colonisers had high growth potential (*K. pneumoniae*, eight copies). Copy number is a proxy for potential rather than realised growth. It was imputed for several colonisers and unavailable for *L. terrae*, and it predicts growth poorly among slow growers [63], so inferences about the surface colonisers are less certain than those about *E. coli*.

Coloniser traits, alternative explanations and biosafety

The dominant colonisers had traits consistent with the chemistry of each system. On the CH biochar, *Paracoccus*, a genus of facultative denitrifiers and methylotrophs reached 66% of the relative abundance. Biochar produced at 400–700 °C can carry redox-active groups that accept and donate electrons [14]. Bacteria can utilise biochar as an electron donor or acceptor [15], which may favour such respiratory taxa. On the CD biochar, proteolytic strict anaerobes (*Proteiniborus*, *Tissierella*, *Sedimentibacter*, *Proteiniphilum*) may have used anoxic microsites and adsorbed proteinaceous substrates.

The biochar was pelleted without washing, so the solid fraction also contained loosely attached cells and residual biochar-liquid. As the liquid fraction was dominated by *E. coli*, its presence would likely reduce the observed differences between the two fractions. Biochar may also adsorb DNA and bias extraction. Low-biomass extracts are vulnerable to reagent contamination [53]: *Burkholderia* and *Ochrobactrum* occurred in three farms, absent in fresh manure and post-incubation without biochar, which is consistent with a medium- or reagent-borne origin, so we do not interpret them as colonisers. However, *Brucella*, the genus into which *Ochrobactrum* has been merged [64], increased in biochar-supplemented manure solids profiled by full-length Nanopore sequencing [65]. *O. intermedium* may also derive from maure populations below our detection limit. *Paracoccus* is also listed among reagent contaminants [53], but *P. koreensis* was confined to one farm and made up 51.4% of a high-biomass sample, which argues against a reagent origin.

The biochar surface also concentrated opportunistic pathogens traceable to the manure, most clearly *K. pneumoniae*, which increased from 1.5% in PH fresh manure to 14.4% on PH biochar; *P. rettgeri* was also enriched on the PH solid relative to its liquid (6.7% vs. 2.3%), although it was less abundant there than in fresh manure (9.6%). Because biochar from manure treatment is often returned to soil, recovered biochar should not be assumed to be microbiologically inert. The 16S rRNA gene cannot establish viability, virulence or antibiotic resistance. Hence, culturing, viability-resolved sequencing and resistome profiling are required to assess risk.

Limitations and future directions

Our study provides a species-level taxonomic view of the chicken manure microbiome and of its selective colonisation of biochar. Nevertheless, several limitations should be considered. First, each production system was represented by one composite sample from one house, so the four groups represent four individual houses rather than replicated housing or feeding treatments. Differences between production systems therefore describe the range of starting communities rather than treatment effects, although the biochar fraction effect was assessed within each unit and was consistent across all four. Second, total ammonia nitrogen and pH were not monitored periodically during incubation because of the small culture volume. The biochar was not characterised by FTIR, SEM or zeta potential, so the chemical mechanisms proposed for surface enrichment remain hypotheses. Besides that, biogas volume, methane and nitrous oxides content were also not measured, so the absence of methanogenic taxa and of methanogenesis-associated functional predictions is an inference from community composition rather than a direct observation of methane production. Finally, sequencing was performed only in fresh manure and at Day 35, so the results describe the endpoint rather than the trajectory of community change. Future studies should include replicate houses within each production category, time-series sampling, gas and ammonia measurements, characterisation of biochar before and after incubation, archaea-inclusive primers or *mcrA* quantification, and qPCR to confirm the activity of key taxa.

Conclusion

Full-length Nanopore 16S rRNA gene sequencing resolved chicken manure communities to species level. The results indicate that biochar acts as a selective filter rather than a uniform driver of community change. The manure entering treatment differed markedly between housing systems. *Lactobacillus* of probable gut origin dominated deep-litter manure, whereas diverse Proteobacteria dominated high-rise manure. During 35 days of anaerobic incubation, the liquid communities converged on facultative Proteobacteria. *E. coli* dominated in PH, PD and CD, and *A. faecalis* in CH. The biochar-associated community remained distinct from this convergence. Across all farms, the biochar-solid carried less *E. coli* and supported higher diversity than the surrounding liquid. Each biochar-solid was also enriched 5- to 76-fold in one or two species that were minor in the biochar-liquid. These colonisers differed between farms. Almost all belonged to species detected on the same farm, so each biochar surface retained the signature of its source manure. Biochar therefore reweighted the local species pool rather than imposing a common community, so the microbial load of recovered biochar depends on the manure it treats. The same surface concentrated opportunistic pathogens, most clearly *K. pneumoniae* which the abundance increased from 1.5% in fresh manure to 14.4% on the biochar of one farm. Recovered biochar should therefore not be assumed to be microbiologically inert before it is returned to soil. Due to each production system was represented by one farm and one bottle, these patterns require confirmation in replicated systems with absolute quantification, viability and resistome analyses and soil studies before recovered biochar is applied to land.

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Competing Interests

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Author Contributions

Zhong Hen Yip: Writing – original draft, Writing – review & editing, Visualization, Validation, Formal Analysis, Data curation, Conceptualization. **Tuan Poy Tee**: Writing – review & editing, Funding acquisition, Supervision, Conceptualization. **Jonathan Long Teng Yeoh**: Writing – review & editing, Validation, Investigation. **Choo Yee Yu**: Writing – review & editing, Validation, Data curation, Investigation. **Mui Yun Wong**: Writing – review & editing. **Geok Hun Tan**: Writing – review & editing. **Nur Hazira Mohd Wazir**: Writing – review & editing. **Siew Ching Tang**: Writing – review & editing, Investigation.

Data Availability

The data that support this study cannot be publicly shared due to ethical and privacy reasons and may be shared upon reasonable request to the corresponding author if appropriate.