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Comparative Profiling of Tissue-Associated Chitinase Signals and Gut Metagenomic Potential in Eight Freshwater Teleost Fishes

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Abstract

Chitin is widespread in aquatic food webs, but its comparative handling among freshwater teleosts is poorly described. Tissue-associated chitinase activity signals, exploratory transcription profiles and gut metagenomic functional potential were characterised across eight freshwater teleost species. Thirteen of 23 metagenomic libraries from six species met the operational classified-read threshold; no library from common carp or black carp was retained, and only one library each was retained for crucian carp and largemouth bass. Assay-derived tissue-associated chitinase activity signals were observed under the present assay conditions for all tissues examined, and the liver-associated sample had the highest observed tissue mean in seven of eight species. The omnibus within-species tissue effect was nominally significant in three species but did not remain significant after false-discovery-rate correction (exact permutation Friedman test, $p = 0.017$–0.028; $q = 0.074$). Exploratory transcription profiling was used as supportive descriptive information. Because RNA input was fixed by volume rather than normalised by mass and the reference gene was not validated across tissues, these data were interpreted cautiously and were not subjected to inferential analysis. The 13 retained metagenomic libraries contained annotations associated with chitin hydrolysis (K01183 and K13381) and downstream amino-sugar metabolism (including nagA, nagB and nagK), together with chitin-related CAZyme families (GH18, GH19, GH20 and CBM5). These annotations indicate predicted functional potential rather than demonstrated microbial activity or dietary chitin utilisation. Between-species patterns were interpreted descriptively because each species was represented by a single aquarium, providing a baseline for future controlled chitin-feeding studies.

Keywords: chitinase; tissue-associated enzyme activity; gut microbiota; CAZyme; chitin-related functional potential; comparative digestive physiology; freshwater teleost

Key Contribution: This study brings together tissue-associated chitinase activity signals and exploratory host transcription profiles with gut metagenomic annotations in a comparative survey of eight freshwater teleost species representing four feeding guilds. It provides a descriptive baseline for future replicated studies; metagenomic interpretation was limited to six species, and the findings do not demonstrate active microbial chitin degradation or species-specific dietary chitin utilisation.

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1. Introduction

Chitin is one of the most abundant natural polysaccharides in aquatic and terrestrial ecosystems and a major structural component of crustacean exoskeletons, insect cuticles, fungal cell walls and many plankton-associated particles [1,2]. For teleost fishes, chitin is encountered through natural prey items, such as zooplankton, aquatic insects and crustaceans, as well as through formulated diets containing insect meal or crustacean-derived ingredients. Insect-derived proteins have attracted increasing attention as sustainable alternatives to fish meal because of their high protein content, favourable resource-conversion efficiency and potential contribution to circular aquaculture systems [3–9]. The nutritional utilisation of insect meals by fish is, however, influenced by chitin content, chitin structure, processing method and host digestive capacity: chitin may act as a fibre-like compound and modulate intestinal microbiota or immune responses at moderate levels, whereas excessive chitin can reduce nutrient digestibility in some species [10–13]. Therefore, understanding variation in chitin-related digestive characteristics among teleost species is important for comparative digestive physiology and may provide ecological and physiological context for interpreting future studies involving chitin-containing feed ingredients. Because teleost species differ in digestive anatomy, physiology and ecological adaptation, their chitin-associated digestive traits may also vary among species. However, comparative information describing these traits across freshwater aquaculture species remains limited.

Insect meals and crustacean processing by-products are among the most actively evaluated alternative protein sources in aquafeeds, and both contain chitin at levels that vary with species, life stage and processing method [3,5,14]. Because chitin is poorly digested by many teleosts, the response to dietary chitin is likely to depend partly on host- and microbiota-associated chitinolytic processes, in addition to ingredient characteristics [10–13]. Comparative information on those processes across farmed species remains fragmentary: most reports involve a single species, a single tissue or a single dietary ingredient, and the assays, substrates and expression normalisation strategies differ between laboratories. A standardised multi-species description may therefore provide useful context for interpreting species-associated patterns reported in feeding trials, even though such a description cannot by itself predict growth or digestibility responses.

The biochemical degradation of chitin in fish is mediated mainly by chitinases, which hydrolyse β-1,4 linkages in chitin and generate chitooligosaccharides and N-acetylglucosamine. Fish chitinases generally belong to glycoside hydrolase family 18 and have been detected in several digestive and accessory digestive tissues, including the stomach, intestine, liver and hepatopancreatic tissue [15,16]. Chitinase activity varies considerably among species and digestive organs, indicating that these characteristics vary with digestive tract morphology, dietary spectrum and tissue-specific enzyme production [17–19]. In gastric fishes, the stomach may contribute to the initial disruption of chitin-containing prey under acidic conditions [2], whereas in agastric or weakly gastric fishes the anterior intestine or hepatopancreatic tissue may contribute more strongly to enzyme secretion and substrate hydrolysis. Enzyme activity alone cannot fully account for chitin-related digestive characteristics, because transcript abundance, enzyme secretion, protein stability, tissue localisation and the contribution of microbial enzymes may all influence the final outcome. Such comparative analyses remain scarce for the species that dominate inland freshwater aquaculture, where herbivorous, omnivorous and filter-feeding fishes are reared alongside carnivorous species.

In addition to host-derived enzymes, the gut microbiota may contribute to the degradation and metabolism of chitin or chitin-derived compounds. The fish gut microbiota is involved in nutrient digestion, intestinal development, energy metabolism, immune regulation and adaptation to dietary substrates [20–26]. Many gut-associated bacteria possess genes encoding carbohydrate-active enzymes (CAZymes) and may participate in the degra-

dation of complex polysaccharides that are otherwise poorly digested by the host; taxa within *Aeromonas*, lactic acid bacteria and polysaccharide-degrading anaerobes have been reported to show chitinolytic or broader polysaccharide-degrading potential [27–29]. Gut microbiota may therefore complement host digestive processes. Microbial composition is, however, strongly influenced by host species, diet, habitat, digestive tract structure and nutrient availability, and the presence of potentially chitinolytic taxa or of predicted metabolic pathways should be interpreted cautiously, because taxonomic composition and functional annotation do not directly demonstrate microbial chitin degradation. Considering gut microbial composition alongside host chitinase activity and exploratory transcription profiles may provide a broader descriptive context for chitin-related traits; however, these parallel observations cannot establish that host and microbial processes are functionally linked or that dietary chitin is degraded in vivo.

Despite this interest, the comparative biochemical basis of chitin-related digestive characteristics among freshwater fishes with contrasting diets remains insufficiently described. The present study therefore examined eight teleost species that are widely cultured in inland China and that span four dietary categories: largemouth bass (*Micropterus salmoides*) and black carp (*Mylopharyngodon piceus*) as carnivorous species; grass carp (*Ctenopharyngodon idella*) and blunt snout bream (*Megalobrama amblycephala*) as herbivorous species; crucian carp (*Carassius auratus*) and common carp (*Cyprinus carpio*) as omnivorous species; and silver carp (*Hypophthalmichthys molitrix*) and bighead carp (*Hypophthalmichthys nobilis*) as filter-feeding species. Tissue-associated chitinase activity was measured in the digestive tissues available in each species, transcription of chitinase genes was profiled within each species by real-time PCR as an exploratory comparison, and gut microbial community composition and chitin-related functional potential were profiled by shotgun metagenomic sequencing. The study aimed to provide a descriptive multi-species framework integrating host-associated enzyme activity with gut microbial functional annotations. The added value of this study is the side-by-side description of tissue-associated chitinase activity signals and exploratory host transcription profiles, together with gut metagenomic functional annotations, in a comparative survey of eight freshwater teleosts representing four feeding guilds. This approach complements reports focused on individual species, tissues, or analytical components by providing a descriptive baseline and identifying candidate chitin-related microbial functions for future testing. Because the qPCR component is exploratory and only 13 metagenomic libraries from six species met the read-depth criterion, these findings are hypothesis-generating and do not establish active chitin degradation or dietary chitin utilisation. Specifically, we addressed three questions: (i) what tissue-associated patterns of chitinase activity are observed within the examined freshwater teleost species; (ii) can exploratory transcription profiling provide descriptive information on tissue-associated variation in chitinase transcription within species; and (iii) do the retained gut metagenomic libraries contain detectable chitin-related functional potential, including genes associated with chitin hydrolysis and downstream N-acetylglucosamine utilisation? Because each species was represented by a single aquarium, between-species patterns were treated as descriptive, and metagenomic results were interpreted only for libraries meeting the predefined read-depth criterion.

2. Materials and Methods

2.1. Ethics Statement

This study was conducted at the Agronomy Experimental Center of the College of Agriculture and Biological Science, Dali University, Yunnan Province, People's Republic of China. All experimental protocols and procedures were approved by the Animal Welfare and Ethical Committee of Dali University (ethics approval code: 2020-PZ-46; approved

on 29 October 2020). Throughout the experimental period, fish were maintained in well-aerated water and handled so as to minimise suffering. All tissue collection and visceral extraction procedures were performed in strict accordance with the Guidelines for the Care and Use of Laboratory Animals in China.

2.2. Experimental Fish, Acclimation and Sampling

The experimental fish, comprising largemouth bass (45.58 ± 3.24 g), black carp (68.06 ± 2.46 g), grass carp (185.12 ± 11.80 g), blunt snout bream (28.50 ± 2.48 g), crucian carp (246.55 ± 5.78 g), common carp (66.40 ± 4.17 g), silver carp (53.77 ± 2.69 g) and bighead carp (50.97 ± 2.46 g), were purchased from Beimen Market, Dali, Yunnan Province, China, and transported to the Aquaculture Laboratory of Dali University. All individuals of a given species were held together in a single aquarium (60 cm × 36 cm × 72 cm), and each aquarium belonged to one species only. Fish were acclimated for 7 days under a standardised regime and fed a common transitional diet (Tables 1 and 2) to reduce short-term dietary and handling-related variation before sampling. The 7-day acclimation period was intended only to minimise short-term handling effects and was not designed to eliminate historical dietary or environmental differences among species. Differences in species origin, body size, developmental stage, prior feeding history and physiological status therefore could not be excluded. Aquaria were supplied with a recirculating water system and continuous aeration, and fish were hand-fed the transitional diet to apparent satiation. Quantitative feed intake and species-specific feeding behaviour were not recorded; therefore, equivalent consumption of the transitional diet by all eight species cannot be confirmed. Feed intake and species-specific feeding behaviour were not quantified systematically. Water quality conditions were maintained according to routine laboratory management procedures. Each aquarium was stocked with individuals of a single species, from which the fifteen sampled individuals were subsequently selected at random.

Table 1. Formulation of the transitional diet (% dry matter).

Ingredients	Proportion (%)
Fish meal	30.00
Soybean meal	22.00
Wheat flour	17.65
Corn gluten meal	5.00
Rapeseed meal	7.00
Peanut meal	6.00
Fish oil	4.00
Soybean oil	3.00
Soy lecithin	2.00
Vitamin premix [1]	1.00
Mineral premix [2]	1.00
Vitamin C	0.05
Choline chloride	0.25
Antioxidant	0.05
Monocalcium phosphate	1.00
Total	100.00

Note: [1] Vitamin premix (per kg diet): vitamin A, 62,500 IU; vitamin D_3, 15,000 IU; vitamin E, 1.75 g; vitamin K_3, 35.4 mg; vitamin B_1, 100 mg; vitamin B_2, 150 mg; vitamin B_6, 150 mg; vitamin B_{12}, 0.2 mg; biotin, 4 mg; D-calcium pantothenate, 500 mg; niacin, 750 mg; folic acid, 25 mg; inositol, 2 g. [2] Mineral premix (per kg diet): $FeSO_4 \cdot H_2O$, 200 mg; $CuSO_4 \cdot 5H_2O$, 96 mg; $ZnSO_4 \cdot H_2O$, 360 mg; $MnSO_4 \cdot H_2O$, 120 mg; $MgSO_4 \cdot H_2O$, 240 mg; KH_2PO_4, 4.2 g; NaH_2PO_4, 0.5 g; KI, 5.4 mg; $CoCl_2 \cdot 6H_2O$, 2.1 mg; Na_2SeO_3, 3 mg.

Table 2. Proximate composition and amino acid profile of the transitional diet (% dry matter).

Items	Content (%)
Proximate composition	
Dry matter	90.95
Crude protein	40.94
Crude lipid	15.08
Ash	13.45
Essential amino acids	
Arginine	2.15
Histidine	0.50
Isoleucine	1.43
Leucine	2.51
Lysine	1.79
Methionine	0.48
Phenylalanine	1.64
Threonine	1.27
Valine	1.67
∑EAA	13.44
Non-essential amino acids	
Aspartic acid	2.44
Serine	2.26
Glutamic acid	5.02
Glycine	2.04
Alanine	1.44
Cysteine	0.30
Tyrosine	1.31
Proline	2.07
∑NEAA	16.88
∑TAA	30.32
∑EAA/TAA (%)	44.33

Note: ∑EAA, total essential amino acids; ∑NEAA, total non-essential amino acids; ∑TAA, total amino acids.

After the acclimation period, fifteen apparently healthy individuals were selected at random from each species. Following deep anaesthesia with 40 mg/L eugenol, fish were euthanised by prolonged immersion in the same anaesthetic solution until opercular movement ceased and no response to tactile stimulation was observed; the digestive tissues available in each species were then collected. The interval between the final feeding and tissue collection was not documented in the available records, so a standardised fasting period could not be confirmed. Tissues from five individuals were combined to form one composite sample, and three composite samples were thereby obtained per tissue per species. Because all individuals of a species came from one aquarium, the three composite samples per species are subsamples of a single aquarium rather than independent biological replicates, and species identity is confounded with aquarium identity. These sampling characteristics were considered in the statistical strategy described in Section 2.8 and the interpretation of all between-species comparisons (Sections 3 and 4). For between-species questions, the aquarium was therefore the experimental unit.

Intestinal contents were collected separately for metagenomic analysis, snap-frozen in liquid nitrogen and stored at −80 °C until DNA extraction.

2.3. Transitional Diet

During the acclimation period, all fish were fed the same transitional diet, which contained no intentionally added purified chitin, insect meal or crustacean-derived chitin-rich ingredient and was intended to provide a common nutritional baseline (Table 1). However, potential endogenous chitin contributed by other feed ingredients was not quantified. The proximate composition and amino acid profile of the diet are given in Table 2.

2.4. Tissue-Associated Chitinase Activity Assay

Chitinase activity was measured in the digestive tissues available in each species. Seven of the eight species are agastric cypriniform fishes and provided esophagus, intestine and liver; largemouth bass, the only gastric species in the set, provided stomach, pyloric caeca, intestine and liver. No stomach tissue exists in the other seven species and none was sampled. The assay was performed with a commercial colorimetric kit (JDZM-1-G, Suzhou Comin Biotechnology Co., Ltd., Suzhou, China).

The intestinal segment was opened and the bulk luminal contents were removed before homogenisation. Because residual microbial and dietary material associated with the mucosal surface could not be excluded, and because the assay is performed on a tissue homogenate, the measured activity may include a contribution from microbial or dietary enzymes; the results are therefore referred to throughout as tissue-associated chitinase activity rather than host chitinase activity.

Briefly, tissue samples were homogenised in ice-cold extraction buffer (1:5–1:10, *w*/*v*) and centrifuged at 10,000× *g* for 20 min at 4 °C. The supernatant was incubated with colloidal chitin at 37 °C for 1 h, and the reaction was terminated by boiling for 5 min. Released reducing sugars were quantified by the 3,5-dinitrosalicylic acid method and absorbance was read at 540 nm on an MB-580 microplate reader (Shenzhen Huisong Technology Development Co., Ltd., Shenzhen, China). One unit (U) of chitinase activity was defined as the amount of enzyme liberating 1 mg of N-acetylglucosamine per hour under the assay conditions. Activity was normalised to tissue protein concentration, determined by the bicinchoninic acid assay, and expressed as U/g protein. Each sample was assayed in three technical replicate wells according to the manufacturer's protocol, and the replicate readings were averaged. Where sample-specific dilution factors were recorded, these were incorporated into the activity calculations. However, differences in tissue-to-buffer ratio, tissue matrix composition and extraction efficiency could not be fully evaluated retrospectively and may have influenced the protein-normalised activity signals. Because complete validation records for substrate and sample blanks, positive controls, assay linearity, and analytical sensitivity were not available for retrospective verification, potential matrix-associated background contributions and the quantitative performance of the assay cannot be fully assessed. Therefore, the measured values are interpreted as tissue-associated chitinase activity signals rather than absolute enzyme activities, and should be considered within the limitations of the present assay conditions.

2.5. RNA Extraction and Quantitative Real-Time PCR

Total RNA was extracted from each tissue sample using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA concentration and purity were assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA), and RNA integrity was evaluated by agarose gel electrophoresis. First-strand cDNA was synthesised from extracted RNA using a commercial reverse transcription kit according to the manufacturer's protocol.

Quantitative real-time PCR was performed using SYBR Green chemistry on a real-time PCR detection system. The amplification programme consisted of an initial denaturation at 95 °C for 5 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 20 s. Melting-curve analysis was conducted from 65 °C to 95 °C (0.5 °C per 5 s) to evaluate amplification specificity. A species-specific actin-family transcript was used as the internal reference; the corresponding transcript annotations are listed in Table 3. Primer sequences, target genes and accession numbers are listed in Table 3. The available primary records did not establish whether no-template controls or no-reverse-transcription controls were included, so their use could not be verified retrospectively.

Table 3. Primers used for quantitative real-time PCR.

Species	Primer	Sequence (5′→3′)	Target Annotation (NCBI)	Accession	Amplicon (bp)	T_a (°C)
Ctenopharyngodon idella	Chitinase-Ci-F	CTGGAGCAGAGTTCTGTGAAGGC	Acidic mammalian chitinase-like (LOC127505034)	XM_051880285.1	150	60
	Chitinase-Ci-R	AGTCGCAGCACAAGCAGTTGG				
Megalobrama amblycephala	Chitinase-Ma-F	ACCTTGCTGTCTGTTGGAGGATTG	Chitinase, acidic.1 (*chia.1*)	XM_048172829.1 [a]	80	60
	Chitinase-Ma-R	GCCTTGCGGTTCTCTGGAGTTG				
Carassius auratus	Chitinase-Ca-F	ACCACTCATGCTCCAGGACCAG	Acidic mammalian chitinase-like (LOC113111355)	XM_026275981.1	145	60
	Chitinase-Ca-R	TGTCATCAAACACGGTGCCTATGG				
Cyprinus carpio	Chitinase-Cc-F	ATTGCTGTTCCTGCGTCTCCATG	Chitinase, acidic.1 (*chia.1*)	XM_042766344.1 [a]	91	60
	Chitinase-Cc-R	CTGTTTGTCTTCTGGTGGGCTTCC				
Micropterus salmoides/*Mylopharyngodon piceus*	Chitinase-Ms-Mp-F	CCCACCTACGGACGAACCTACC	Chitinase, acidic.1 (*chia.1*)	XM_038697474.1 [a,b]	107	60
	Chitinase-Ms-Mp-R	CAGAAGCCAGCAGTGCGAGTG				
Hypophthalmichthys nobilis/*H. molitrix*	Chitinase-Hn-Hm-F	GCTAACCGCCAGACCTTCATCAC	Acidic mammalian chitinase-like (LOC134461936)	XM_063214755.1 [b]	177	60
	Chitinase-Hn-Hm-R	CTTCGTACCCTCAGCCTCAAATGC				
Ctenopharyngodon idella	β-actin-Ci-F	CGTGCTGTCTTCCCATCCATCG	Actin, beta 2 (*actb2*)	XM_051886219.1	96	60
	β-actin-Ci-R	CTGAGCCTCGTCACCAACATAGC				
Megalobrama amblycephala	β-actin-Ma-F	GCTGGATTCGCTGGAGATGATGC	Actin, beta 2 (*actb2*)	XM_048192430.1	123	60
	β-actin-Ma-R	CTGAGCCTCGTCACCAACATAGC				
Carassius auratus	β-actin-Ca-F	TCACCTCCCTTGCTCCATCCAC	Actin, cytoplasmic 1 (LOC113044540)	XM_026204620.1	134	60
	β-actin-Ca-R	CTCCTGCTTGCTGATCCACATCTG				
Cyprinus carpio	β-actin-Cc-F	CCACCTCCCTTGCTCCCTCCAC	Actin, beta 1 (*actb1*)	XM_019089433.2	134 [c]	60
	β-actin-Cc-R	CTCCTGCTTGCTGATCCACATCTG				
M. salmoides/*M. piceus*	β-actin-Ms-Mp-F	ATCGTGCGTGACATCAAGGAGAAG	Actin, beta 2 (*actb2*)	XM_038695351.1 [b]	81	60
	β-actin-Ms-Mp-R	GGAGGAGGAGGCAGCGGTAC				
H. nobilis/*H. molitrix*	β-actin-Hn-Hm-F	TGACAGAGGCTCCACTCAACCC	Actin, epsilon 1 (*acte1*)	XM_063196941.1 [b]	156	60
	β-actin-Hn-Hm-R	CACCAGCGTCCAGCACAATACC				

Note: Ta, annealing temperature; every primer set was run at 60 °C. Amplicon lengths were calculated in silico from the positions of the primer binding sites on the reference transcript listed in the same row; they were not verified by electrophoresis or by sequencing the amplicons. [a] The accession given in the original primer record did not contain either primer site and was replaced by the transcript of the same species in which both sites occur. [b] Primer set shared by two species; the amplicon length refers to the transcript of the species named first, and no transcript of the second species containing both primer sites was identified, so amplicon identity in the second species could not be independently confirmed: a single melting-curve peak indicates predominant amplification of one product but does not establish its sequence identity, and the amplicons were not sequenced. [c] One mismatch between the forward primer and the listed reference transcript. Amplification efficiency and the coefficient of determination (R^2) of a standard curve are not reported because no standard curves were run; see Section 2.5. Species-specific actin-family transcripts were used as reference targets, but their stability across tissues was not validated; variation in the corresponding reference-transcript Ct values was examined descriptively within each species and is reported in Table S7 (labelled β-actin Ct in the original assay records), together with a sensitivity analysis performed without reference-gene normalisation.

Given that amplification efficiency calibration and amplicon sequencing were not performed, qPCR results were interpreted as exploratory within-species transcription profiles rather than quantitative comparisons of absolute transcript abundance.

For each species, three composite samples were analysed, and each composite sample was measured using three technical replicates. Technical replicate Ct measurements were averaged before subsequent analyses, and composite samples were considered the analytical units. Relative transcription was calculated using the ΔCt method (Ct_target gene − Ct_reference transcript). For each species, the tissue with the lowest mean ΔCt across the three composite samples was used as the calibrator and assigned a relative expression value of 1. Relative expression estimates were calculated as $2^{-\Delta\Delta Ct}$ (ΔΔCt = mean ΔCt of the tissue—mean ΔCt of the calibrator tissue), following Livak and Schmittgen [30], and are presented in Figure S4 and Table S8.

Because RNA input was normalised by volume rather than RNA mass, and because the stability of the actin-family reference transcript across all examined tissues and species was not independently validated, the resulting transcription patterns were interpreted cautiously. No additional candidate reference genes were evaluated, and the available records did not establish whether no-template or no-reverse-transcription controls were included. These limitations prevent the qPCR results from being treated as confirmatory evidence. No inferential statistical analysis was applied to the qPCR dataset.

Primer sequences were designed based on available reference transcripts for each species. For two species pairs (largemouth bass, *Micropterus salmoides*, and black carp, *Mylopharyngodon piceus*; bighead carp, *Hypophthalmichthys nobilis*, and silver carp, *H. molitrix*), shared primer sets were used on the basis of the available reference-sequence information. For the second species in each pair (black carp and silver carp), no reference transcript containing both primer-binding sites was identified; therefore, amplification of the intended target in these two species cannot be confirmed. Predicted amplicon sizes were obtained through in silico alignment with the reference transcripts listed in Table 3, and melting-curve analysis indicated predominant single amplification products. Because amplicon identity was not independently confirmed by sequencing, these qPCR results are presented as descriptive observations rather than definitive transcript-level evidence.

2.6. Metagenomic Sequencing, Quality Control and Taxonomic Profiling

Three pooled intestinal content samples per species were subjected to paired-end (2 × 150 bp) shotgun metagenomic sequencing on the Illumina NovaSeq platform with a 350 bp insert, giving twenty-three libraries in total (three per species, except grass carp, for which two libraries were available; no sequence data were obtained for the third pooled sample of that species). Genomic DNA was extracted from intestinal contents by the sequencing provider with a commercial kit following the manufacturer's instructions, and the extracted DNA was checked before library construction; libraries were prepared with the provider's standard paired-end library preparation kit and were verified on an Agilent 2100 Bioanalyzer and by quantitative PCR, as documented in the provider's experimental workflow. The identity of neither the extraction kit nor the library preparation kit is stated in the project report.

Raw reads were first processed with KneadData, which performs adapter and quality trimming with Trimmomatic v0.39 [31] (ILLUMINACLIP:2:30:10, SLIDINGWINDOW:4:20, MINLEN:50) and removes host-derived reads by alignment with Bowtie2 v2.3.5.1 [32] (–very-sensitive). Read quality was inspected with FastQC both before and after this step, and the same trimming and host-removal procedure was applied to all twenty-three libraries. The host reference database supplied by the provider for the removal step was not documented in the project report. Host-read retention differed substantially among libraries;

because reference-genome completeness differs among the eight species, the extent to which this variation reflects reference choice or sample characteristics cannot be determined. The retained fraction is reported per library in Table S1, and the missing documentation is noted as a limitation in Section 4.4.

Taxonomic profiling was performed with Kraken2 v2.0.7-beta [33] at a confidence threshold of 0.2 against a database built from bacterial, archaeal, fungal and viral sequences in NCBI NT and RefSeq. The build date and exact release of the Kraken2 reference database were not documented in the provider's project report, which limits exact reproducibility of the taxonomic assignments, and species-level abundances were re-estimated with Bracken v2.0 [34] using its default parameters. Relative abundances were summarised across taxonomic levels and expressed as the proportion of classified reads. Alpha diversity indices (observed richness, Chao1, ACE, Shannon, Simpson, inverse Simpson, Pielou's evenness and Good's coverage) were computed from the species-level profile.

Sequencing and read-retention statistics for every library, including raw reads, clean reads, clean-read percentage, sequence quality, classified reads at species level and fungal read proportion, are provided in Table S1. Sequencing depth was strongly bimodal overall, although one retained library (AN3) had an intermediate depth of 236,818 classified reads. Ten libraries had fewer than 16,400 classified reads at the species level, while the thirteen libraries retained for downstream taxonomic and functional interpretation had 0.237–10.24 million classified reads. AN3 exceeded the operational threshold of 50,000 classified reads and was retained in downstream analyses; its lower depth may nevertheless reduce sensitivity for low-abundance annotations. The threshold separates the ten low-depth libraries ($\leq$16,399 classified reads) from the lowest retained library (236,818 classified reads). The ten libraries below the threshold also showed a lower raw-library GC content and a higher fungal fraction among the retained reads, and yielded almost no carbohydrate-active enzyme annotation (Section 3.5); they are reported in Table S1 but were excluded from functional interpretation.

2.7. Functional Profiling: KEGG, CAZy and Chitin-Related Targets

Functional profiling was performed with HUMAnN3 v3.6 [35], in which translated reads are aligned with DIAMOND v0.9.14 [36] against the UniRef90 database [37] and quantified as gene families; the gene-family table is then mapped to reference databases through the UniRef90 identifier. KEGG Orthology (KO) assignments, KEGG pathways and KEGG modules were obtained with the corresponding utility mappings distributed with HUMAnN3 [38]. CAZy families [39] were generated by applying the same grouping logic to the same gene-family table, through a UniRef90-to-CAZy correspondence supplied with the provider's pipeline. CAZy is not among the utility mappings distributed with HUMAnN3, and neither the construction of that correspondence—whether it derives from dbCAN annotation, from DIAMOND alignment against CAZy sequences, or from a curated UniRef90-to-CAZy list—nor its version is documented in the project report, and it was not available for inspection. The releases of all mapped databases were those bundled with the provider's HUMAnN3 v3.6 database build, and the release dates are likewise not documented. Because the provenance of the CAZy mapping was not available in the provider documentation, CAZy results are interpreted only at family level, and no inference is drawn about subfamily assignment, module architecture or substrate specificity; the same reservation applies to the chitin-related families listed below and reported in Tables S2 and S6. For the same reason the CAZy profiles are treated throughout as exploratory supporting evidence, whereas the KEGG orthologue profiles provide the primary annotation-based functional evidence used in this study, because their mapping route is documented within the standard HUMAnN3 workflow, although the unavailable database release dates limit

exact computational reproducibility. Gene-family abundances were renormalised to copies per million (CPM) with the HUMAnN3 utility script 'humann_renorm_table –units cpm'. All CAZy-family and KO abundances reported in this manuscript, in Tables S2, S3 and S6, are therefore in CPM; the column sums of the reported tables fall below 10^6 because only the subset of gene families annotated to the database in question is shown.

Because the objective of this study concerns chitin, the functional analysis focused on (i) CAZy families acting on chitin and chitin oligomers—GH18, GH19, GH20, CBM2, CBM5, CBM12, GH85 and AA10—and (ii) KEGG orthologues associated with chitin hydrolysis and downstream amino-sugar metabolism: K01183 (chitinase, EC 3.2.1.14), K13381 (bifunctional chitinase/lysozyme), K01207 (β-N-acetylhexosaminidase), K12373 (hexosaminidase), K18676 (glucosamine kinase), K01443 (N-acetylglucosamine-6-phosphate deacetylase), K02564 (glucosamine-6-phosphate deaminase) and K00884 (N-acetylglucosamine kinase). K01183 and K13381 are directly associated with chitin hydrolysis, whereas the remaining KOs are associated with hexosaminidase-related processing or downstream amino-sugar metabolism and are not, by themselves, specific evidence of chitin degradation. Family- and orthologue-level abundances were normalised as implemented in HUMAnN3 and are reported per library in Tables S2 and S3.

2.8. Statistical Analysis

Data are presented as mean ± standard deviation. For between-species questions, the aquarium was the experimental unit. Because each species was represented by only one aquarium, there was no independent replication at the species level and species and aquarium effects could not be separated. The three composite samples were therefore treated as within-aquarium subsamples rather than as independent species-level replicates. The statistical strategy reflects this design.

(i) Within-species tissue comparisons. The same composite samples provided several tissues, so tissues are not independent observations. Tissue differences within a species were therefore tested with an exact permutation Friedman test, a paired non-parametric test appropriate for a randomised-block design with the composite sample as the blocking factor. Because only three blocks were available, the asymptotic χ^2 approximation of the Friedman statistic is unreliable and *p*-values were obtained from the permutation distribution of the statistic instead: the rank matrix was enumerated over all (k!)^b arrangements of the ranks within blocks, where k is the number of tissues and b the number of composite samples. Chitinase activity was measured in three tissues in the seven cypriniform species and in four tissues in largemouth bass (Section 2.4), giving 216 arrangements at k = 3 and 13,824 at k = 4, so exact enumeration was feasible for all eight species and no Monte-Carlo approximation was required. No inferential test was performed for the exploratory qPCR component (see (v)); no permutation procedure was therefore applied to the transcription data and no random seed arises anywhere in this manuscript. The asymptotic *p*-value is reported alongside the permutation *p*-value in Table S4 for transparency. For the same reason of sample size, the smallest attainable two-sided *p*-value of a Wilcoxon signed-rank post hoc test is 0.25; pairwise post hoc comparisons are therefore not informative in this dataset and are not reported, and no post hoc lettering is used in any figure.

(ii) Between-species comparisons. Because species and aquarium are confounded and no species-level replication exists, between-species comparisons of chitinase activity and of transcription are reported descriptively only; no inferential statistics and no *p*-values are presented for any comparison of the same tissue among species. Statements referring to a rank order refer to the observed order of means within the present dataset.

(iii) Gut microbial community structure Bray–Curtis dissimilarities were computed from the species-level profiles and visualised by principal coordinates analysis (PCoA) with

the vegan package in R [40]. Because each species was represented by a single aquarium, the three libraries of a species are subsamples of one aquarium and a permutational multivariate analysis of variance [41] would estimate a species effect that this design cannot support [42]; no inferential test of a species effect on community structure was therefore performed and no *p*-value is reported for the ordination. The ordination is used as an exploratory description of how the retained libraries are positioned relative to one another.

(iv) Chitin-related functional potential. Family- and orthologue-level abundances are reported per library and, for species with at least two libraries, as means. No between-species statistical test is applied to CAZyme or KO abundances, because of the confounding described above and the very uneven retained microbial read depth among libraries. Alpha diversity indices are reported per library together with their depth statistics (Table S1) and are not formally compared among species, for the same reason. Library-level variables are described rather than tested in the same way. Raw-library GC content is reported for every library in Table S1 and summarised by group, but no inferential test is applied to it: the two groups are defined by the retained classified microbial read depth after host-read removal, so any test comparing them would be circular, and the comparison is used only to describe how the excluded libraries differ.

(v) Multiple testing. One Friedman test was performed per species for chitinase activity, giving a single family of eight tests. *p*-values were adjusted within this family with the Benjamini–Hochberg procedure to control the false discovery rate [43]. Both the uncorrected *p* and the adjusted q are reported in Table S4 and in the text. No inferential test was performed for the exploratory qPCR component: no Friedman test, no *p*-value, and no *q*-value were calculated for transcription, and the supplementary qPCR tables contain descriptive Ct/ΔCt and relative-expression summaries only. A result is described as significant only where $q < 0.05$; where $p < 0.05$ but $q \geq 0.05$, the result is described as nominally significant and is not treated as a finding.

Differences are described as significant only when the Benjamini–Hochberg-adjusted *q*-value of a within-species Friedman test is below 0.05; no other inferential test is reported anywhere in this manuscript. Figures were prepared with R 4.6.1 using the ggplot2 and vegan packages; error bars represent standard deviation.

3. Results

3.1. Tissue Distribution of Tissue-Associated Chitinase Activity Within Species

Assay-derived tissue-associated chitinase activity signals were observed in every digestive tissue examined across the eight species, with tissue means ranging from 4.39 ± 0.02 to 11.44 ± 3.98 U/g protein (Figure 1, Tables 4 and S4). Because the same composite samples provided multiple tissues, within-species tissue differences were analysed using a paired Friedman test.

Hepatic activity gave the highest observed tissue mean in seven of the eight species (black carp, blunt snout bream, common carp, crucian carp, grass carp, largemouth bass and silver carp), exceeding the corresponding intestinal value by factors of 1.3 to 2.4. The omnibus Friedman test for differences among tissues was nominally significant in three species: largemouth bass ($Q = 8.20$, $p = 0.017$), blunt snout bream ($Q = 6.00$, $p = 0.028$) and crucian carp ($Q = 6.00$, $p = 0.028$). After Benjamini–Hochberg correction across the eight species, all three had $q = 0.074$, so no species remained significant at $q < 0.05$ and the omnibus tissue effect in chitinase activity is reported here as nominally significant only. In black carp, common carp, grass carp and silver carp, the liver-associated mean was likewise the highest observed tissue mean, although the omnibus tissue effect was not significant ($Q = 4.67$, $p = 0.194$, $q = 0.222$ in all four). In bighead carp, no omnibus tissue effect was

detected (Q = 0.67, $p = 0.944$, $q = 0.944$), with the intestinal mean (5.16 ± 0.38 U/g protein) slightly exceeding the hepatic mean (4.99 ± 0.30 U/g protein).

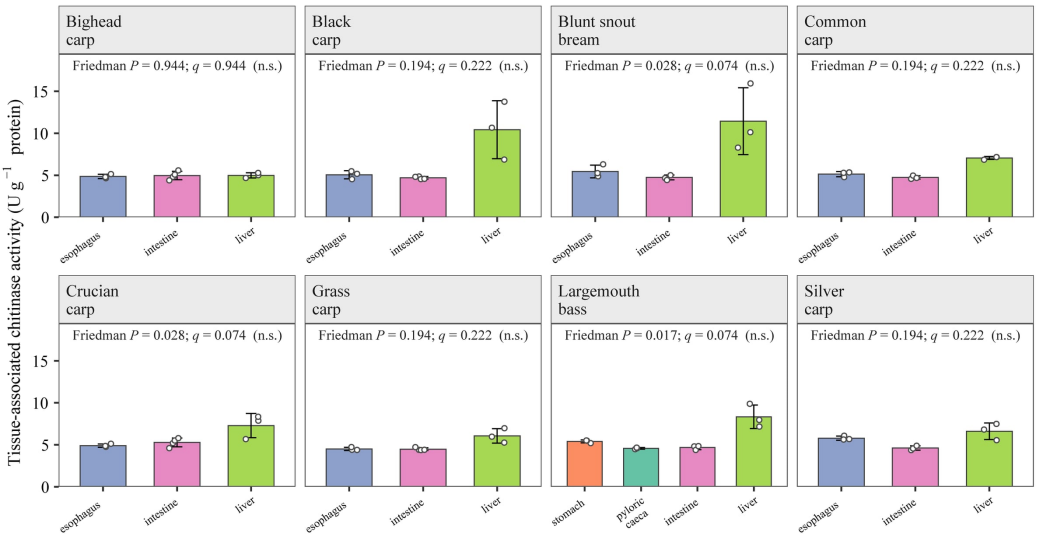


Figure 1. Tissue-associated chitinase activity within each species. Bars represent the mean of three within-aquarium composite subsamples; error bars indicate SD; open circles represent individual composite subsamples. These subsamples are not independent aquarium-level biological replicates. *p*-values are nominal Friedman test results and *q*-values are Benjamini–Hochberg-adjusted across the eight species; no species remained significant after correction ($q < 0.05$).

Table 4. Tissue-associated chitinase activity (U/g protein, mean ± SD, $n = 3$ within-aquarium composite subsamples) in eight freshwater teleost species, with the exact permutation Friedman test result for tissue differences within each species.

Species	Esophagus	Stomach	Pyloric Caeca	Intestine	Liver	Friedman Q	*p* (Exact)	*q* (BH)
Bighead carp	4.86 ± 0.26	—	—	5.16 ± 0.38	4.99 ± 0.30	0.67	0.944	0.944
Black carp	5.06 ± 0.49	—	—	4.66 ± 0.17	10.43 ± 3.45	4.67	0.194	0.222
Blunt snout bream	5.45 ± 0.76	—	—	4.74 ± 0.29	11.44 ± 3.98	6.00	0.028	0.074
Common carp	5.14 ± 0.31	—	—	4.74 ± 0.20	7.06 ± 0.18	4.67	0.194	0.222
Crucian carp	4.90 ± 0.20	—	—	5.52 ± 0.27	7.29 ± 1.44	6.00	0.028	0.074
Grass carp	4.51 ± 0.19	—	—	4.39 ± 0.02	6.07 ± 0.86	4.67	0.194	0.222
Largemouth bass	—	5.40 ± 0.18	4.58 ± 0.09	4.68 ± 0.25	8.33 ± 1.40	8.20	0.017	0.074
Silver carp	5.78 ± 0.25	—	—	4.62 ± 0.26	6.61 ± 0.98	4.67	0.194	0.222

Note: "—" indicates that the tissue was not present in that species: the seven cypriniform species are agastric and have no stomach, and pyloric caeca were assayed in largemouth bass only. The same three composite samples provided all tissues within a species, so tissues were compared with the Friedman test (paired); *p*-values come from the permutation distribution of the Friedman statistic, obtained by exact enumeration of the rank arrangements within blocks (216 arrangements at three tissues, 13,824 at four tissues); *q*-values are Benjamini–Hochberg adjusted across the eight species. At $n = 3$ the smallest attainable two-sided *p*-value of a Wilcoxon signed-rank post hoc test is 0.25, so pairwise post hoc comparisons are not informative and are not reported. No value reaches $q < 0.05$; the three species with $p < 0.05$ are nominally significant only.

The observed magnitude of the enzymatic signal varied across the sampled species. Hepatic activity ranged from 4.99 ± 0.30 U/g protein in bighead carp to 11.44 ± 3.98 U/g protein in blunt snout bream and 10.43 ± 3.45 U/g protein in black carp, whereas intestinal activity was much less variable across species (4.39 ± 0.02 to 5.52 ± 0.27 U/g protein). Crucian carp showed the highest intestinal mean of the eight species (5.52 ± 0.27 U/g protein), followed by bighead carp (5.16 ± 0.38 U/g protein).

Because three composite samples per species were analysed, pairwise post hoc comparisons are not informative (the smallest attainable two-sided *p*-value of a Wilcoxon

signed-rank test at $n = 3$ is 0.25) and no post hoc lettering is used in Figure 1. The Friedman analysis describes the consistency of tissue rankings across the three composite subsamples within each aquarium; because no test remained significant after FDR correction, no tissue effect is considered statistically supported at the adjusted significance level. Because no informative pairwise post hoc comparison was possible at $n = 3$, these omnibus results do not establish a statistically significant difference between hepatic and intestinal activity.

3.2. Descriptive Between-Species Patterns of Tissue-Associated Chitinase Activity

Tissue-associated chitinase activity patterns among species are presented descriptively (Figure 2, Table 4). Because species identity was confounded with aquarium identity, these observations are interpreted as descriptive patterns rather than statistical comparisons.

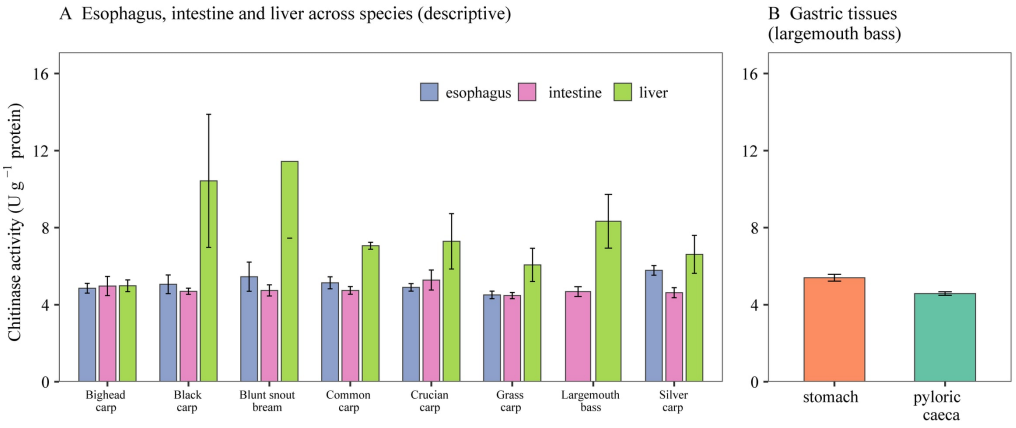


Figure 2. Tissue-associated chitinase activity in digestive tissues of eight freshwater teleost species. (**A**) Activity in the intestine and liver of all eight species and in the esophagus of the seven cypriniform species. (**B**) Activity in the stomach and pyloric caeca of largemouth bass. Bars represent the mean of three within-aquarium composite subsamples, and error bars indicate SD. Each species was represented by a single aquarium, so between-species comparisons are descriptive.

Liver-associated activity showed a much wider spread among species than intestinal activity: hepatic means ranged from 4.99 ± 0.30 U/g protein in bighead carp to 11.44 ± 3.98 U/g protein in blunt snout bream, whereas intestinal means fell within a narrow band (4.39 ± 0.02 to 5.52 ± 0.27 U/g protein). The stomach (5.40 ± 0.18 U/g protein) and the pyloric caeca (4.58 ± 0.09 U/g protein) were available in largemouth bass only. Species means for every tissue are listed in Table 4 and shown in Figure 2.

Within that overall pattern, the ordering of species differed among tissues: crucian carp showed the highest intestinal mean, whereas blunt snout bream and black carp showed higher hepatic means.

3.3. Exploratory Profiling of Chitinase Gene Transcription Within Species

Chitinase gene transcription was profiled within each species as an exploratory and supportive component. Chitinase transcription profiles showed tissue-associated variation within the examined species and were presented as descriptive patterns. The three technical replicates of each composite sample were averaged before calculation, so each tissue is represented by three within-aquarium composite subsamples. ΔCt values varied among tissues in all examined species, and the tissue showing the lowest mean ΔCt differed among species. The relative ranking of tissues differed between the ΔCt-based analysis and the sensitivity analysis based on unnormalised target Ct values; therefore, no single tissue was consistently identified as having the lowest transcription signal across species. The ΔCt values are provided in Table S5, while relative expression estimates are presented in Table S8

and Figure S4. For black carp (*Mylopharyngodon piceus*) and silver carp (*Hypophthalmichthys molitrix*), the corresponding transcription profiles are shown as exploratory observations.

3.4. Relation Between Transcription and Tissue-Associated Enzyme Activity

Transcription and tissue-associated enzyme activity were assessed at different anatomical resolutions. Enzyme activity was measured using whole-intestine samples, whereas transcriptional profiles were obtained separately from the foregut, midgut and hindgut. Therefore, the two datasets were interpreted as complementary descriptive measurements rather than directly equivalent variables.

The exploratory transcription profiles showed tissue-associated variation in several species (Section 3.3). However, these patterns were not used to assign dominant transcription sites or to establish species-specific relationships between transcriptional profiles and tissue-associated enzyme activity. The potential reasons for the lack of parallel patterns between the two measurements are discussed in Section 4.2.

3.5. Sequencing Depth and Data Availability of the Metagenomic Libraries

Twenty-three libraries were sequenced, with raw reads ranging from 19.6 to 49.7 million per library (Table S1). Host-read removal had very different consequences among libraries. In thirteen libraries, 14.3–95.6% of raw reads were retained and 0.237–10.24 million reads were classified at species level. In the remaining ten libraries, only 0.37–3.03% of raw reads were retained, and 1319–16,399 reads were classified at the species level. These ten libraries did not meet the operational threshold of 50,000 classified reads and were excluded from downstream functional interpretation. In these ten libraries, the fungal fraction of the retained reads was 5.3–71.4%, compared with 0.03–8.58% in the thirteen retained libraries (all but one below 1.15%), and the dominant fungal taxa were *Vanderwaltozyma polyspora*, *Kluyveromyces marxianus*, *Lodderomyces elongisporus*, *Saccharomyces cerevisiae*, *Malassezia restricta*, *Lasiodiplodia theobromae* and *Alternaria alternata*, together with bacterial genera that may occur in environmental or reagent-associated contexts. No CAZy family was annotated in eight of these ten libraries, and none of the chitin-related CAZy families was annotated in nine of them.

The ten excluded low-depth libraries also differed from the retained libraries in the base composition of the sequenced DNA: raw GC content was 37–43% (median 38%) in the excluded libraries and 39–60% (median 50%) in the retained libraries. The fungal fraction of classified reads was higher in the excluded libraries (median 20.9%, range 5.3–71.4%) than in the retained libraries (median 0.19%, range 0.03–8.58%). These differences were used only to describe the characteristics of low-depth libraries and were not subjected to statistical comparison. The fungal fractions of the excluded libraries are also proportions of very few classified reads (1319–16,399) and are correspondingly imprecise. The per-library values underlying this comparison are given in Table S1.

These ten libraries were therefore excluded from downstream functional interpretation because of insufficient classified microbial read depth. The retained library set used for downstream functional interpretation consisted of thirteen libraries from six species. Data availability at species level was therefore as follows (Figure 3, Table S1): silver carp 3/3, bighead carp 3/3, blunt snout bream 3/3, grass carp 2/2, crucian carp 1/3, largemouth bass 1/3, common carp 0/3 and black carp 0/3. With a threshold of 50,000 classified reads, thirteen libraries from six species were retained for interpretation.

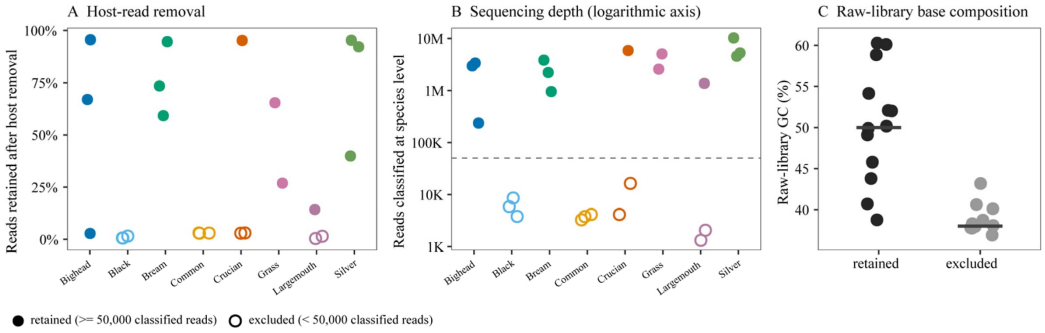


Figure 3. Sequencing depth, data availability and base composition of the 23 metagenomic libraries. Note: Open symbols in panels (**A**,**B**): the ten libraries excluded from functional interpretation (<50,000 classified reads; dashed line in panel (**B**). Table S1 identifies the retained or excluded status of each library. In panel (**C**) the horizontal bar is the median of each group; the two groups are compared descriptively only and no statistical test is applied.

3.6. Gut Microbial Community Structure

Compositional variation was visualised by principal coordinates analysis (PCoA) based on the Bray–Curtis dissimilarity matrix. Figure 4A presents all twenty-three libraries to illustrate the distribution of libraries before quality filtering, whereas Figure 4B shows the thirteen retained libraries used for downstream interpretation. The ordination was used to visualise compositional relationships among retained libraries, and no statistical test was applied to the PCoA.

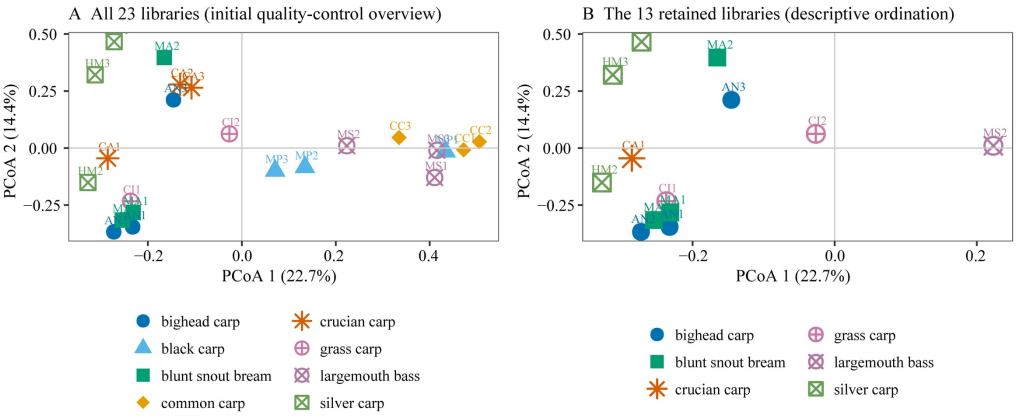


Figure 4. Gut microbial community structure visualised by principal coordinates analysis (PCoA) based on Bray–Curtis dissimilarities. (**A**) Ordination of all 23 metagenomic libraries for quality assessment, including the 10 libraries excluded from downstream interpretation because of insufficient classified microbial read depth. (**B**) Ordination of the 13 retained libraries. Each point represents one library, and colours and shapes indicate the source fish species. Table S1 lists the retention status of each library. The ordination is descriptive, with one aquarium per species.

The first two PCoA axes accounted for 22.7% and 14.4% of the variation, respectively. The ten low-depth libraries displayed distinct positions in the ordination space (Figure 4A), consistent with their different sequencing characteristics. Because these libraries contained insufficient classified microbial reads, they were shown only to illustrate the quality-control process and were not considered in downstream ecological interpretation.

Among the thirteen retained libraries, several compositional patterns were observed. Silver carp and bighead carp libraries occupied different regions of the ordination space, whereas the two grass carp libraries showed relatively similar positions. The retained

libraries from filter-feeding species displayed partial separation from some other taxa. These patterns represent exploratory variation among retained libraries.

3.7. Taxonomic Composition of the Gut Microbiota

Taxonomic composition was summarised for the thirteen retained libraries with adequate classified microbial read depth and is reported in full in Figures S1 and S2 and Table S1; only the features relevant to the chitin-related interpretation are given here. Pseudomonadota was the predominant phylum in most retained libraries, accounting for 28–97% of classified reads, followed by Actinomycetota (1–62%) and Bacillota (0.4–65%). Bacteroidota showed marked variation among libraries, reaching 42% in one grass carp library while remaining below 2% in most other libraries. Phylum-level profiles varied among and, to a considerable extent, within species.

At genus level, the most abundant taxa across the retained libraries were *Vibrio* (mean 12.6%), *Microbacterium* (9.9%), *Aeromonas* (5.5%), *Bosea* (4.9%) and *Aurantimicrobium* (4.7%); the full genus-level inventory is given in Figure S2 and Table S1. Several abundant genera detected in the retained libraries have been reported from aquatic and environmental-associated habitats; therefore, genus-level profiles are presented as descriptive inventories of the examined libraries. Species represented by fewer retained libraries are presented only at the library level. Alpha diversity indices are reported per library in Table S1; given the variation in retained library numbers among species and the limited representation of some species, alpha diversity indices were not compared statistically among species.

3.8. Chitin-Related CAZyme Families and KEGG Orthologues

To characterise chitin-related functional potential, analyses focused on CAZy families associated with chitin degradation and KEGG orthologues involved in chitin hydrolysis and N-acetylglucosamine metabolism (Figure 5, Tables S2 and S3). Among the thirteen retained gut metagenomic libraries, several chitin-associated CAZy families were detected, including glycoside hydrolase families GH18, GH19 and GH20, as well as carbohydrate-binding modules CBM5 and CBM12. These CAZy families were present across multiple retained libraries, although their relative abundance varied among libraries. Additional chitin-associated carbohydrate-active enzyme families, including GH85 and AA10, were also detected in some libraries.

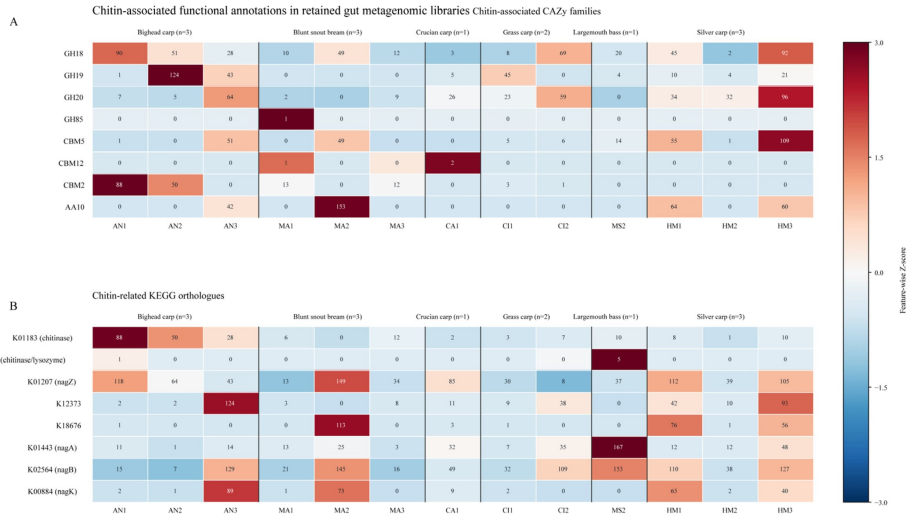


Figure 5. (**A**) Chitin-associated CAZy families and (**B**) KEGG orthologues detected in retained gut metagenomic libraries. Each column represents one retained library; libraries are grouped by species and displayed individually without averaging or clustering. Cell colour indicates the feature-wise Z-

score calculated across all 13 retained libraries, whereas the overlaid numbers represent untransformed CPM rounded to the nearest integer. Thus, colour and numerical values represent different scales. Library codes correspond to the source species: AN, *Aristichthys nobilis*; MA, *Megalobrama amblycephala*; CA, *Carassius auratus*; CI, *Ctenopharyngodon idella*; MS, *Micropterus salmoides*; HM, *Hypophthalmichthys molitrix*. The number of retained libraries varied among species because libraries that did not meet the predefined microbial read-depth criterion were excluded. No statistical comparisons were performed. These results represent annotation-based estimates of predicted functional potential rather than direct measurements of enzyme activity.

The abundance profiles of chitin-associated CAZy families showed library-level variation among the analysed samples. GH18 was detected across the retained libraries, whereas other CAZy families displayed more restricted distributions. The observed patterns indicated differences in the representation of chitin-associated carbohydrate-active enzyme annotations among the retained metagenomic libraries.

KEGG annotation identified eight orthologues associated with chitin hydrolysis or downstream amino-sugar metabolism (Figure 5, Table S3). K01183 (chitinase) and K13381 (bifunctional chitinase/lysozyme) are directly associated with chitin hydrolysis. K01207 (β-N-acetylhexosaminidase) and K12373 (hexosaminidase) are associated with hexosaminidase-related processing, whereas K18676 (glucosamine kinase), K01443 (N-acetylglucosamine-6-phosphate deacetylase), K02564 (glucosamine-6-phosphate deaminase) and K00884 (N-acetylglucosamine kinase) are associated with downstream amino-sugar metabolism. These annotations indicate predicted functional potential and do not demonstrate gene expression, enzyme activity or in vivo chitin degradation.

Because these results are based on metagenomic annotation, they represent predicted functional potential rather than direct measurements of enzyme activity or microbial expression.

4. Discussion

4.1. Species- and Tissue-Level Variation in Tissue-Associated Chitinase Activity

Chitin is a structural polysaccharide that enters the digestive tract of freshwater fishes through zooplankton, aquatic insects, benthic invertebrates, fungal particles and detrital organic matter, and it is also present in formulated diets containing insect meal or crustacean by-products [1,2]. In the present study, assay-derived tissue-associated chitinase activity was measurable in every digestive tissue examined across the eight species, consistent with earlier reports of chitinase activity in the digestive tract of teleosts as different as Atlantic cod and rainbow trout [15,17,18]. Whether this assay-derived signal translates into effective dietary chitin utilisation remains to be determined.

The distribution of that activity across tissues was not uniform. Hepatic activity gave the highest observed tissue mean in seven of the eight species and exceeded the corresponding intestinal value by a factor of 1.3–2.4; the only species in which intestinal activity was marginally the highest was bighead carp. In teleosts, the liver and the exocrine pancreas are anatomically closely associated, and the exocrine pancreas is a principal source of the digestive enzymes that act in the intestine [44]. The samples assayed here were dissected as hepatic tissue and were not examined histologically, so adherent pancreatic tissue cannot be excluded and the measured activity cannot be assigned to hepatic parenchyma alone. The relatively high activity detected in liver-associated samples may therefore reflect enzymes present in the sampled tissue-associated fraction, including possible contributions from adherent pancreatic tissue, or from both hepatic and pancreatic-associated sources, while the pancreatic component remains a plausible but untested contributor. Intestinal tissue showed detectable activity in all eight species, and stomach tissue from the only gastric

species, largemouth bass, also showed detectable activity; in both cases the level was below the corresponding hepatic mean.

Two features of these data qualify how the results should be used. First, the omnibus difference among tissues was nominally significant in three of the eight species only, and none of the three remained significant after correction for multiple testing ($q = 0.074$). With three composite samples per species, the paired test has limited power. A similar general pattern, in which the liver-associated mean was the highest observed tissue value, was also present in black carp, common carp, grass carp and silver carp but was not statistically supported ($p = 0.194$, $q = 0.222$). The tissue distribution of chitinase activity should therefore be read as a recurrent descriptive pattern observed in seven of the eight species for which statistical support is weak, rather than as a demonstrated tissue effect. Reporting the full set of eight tests, as done here, is more informative than reporting only the significant subset, but it also means that the tissue distribution of chitinase activity cannot be described as a uniform property of these fishes. Second, the between-species range of hepatic activity (4.99–11.44 U/g protein) was much wider than that of intestinal activity (4.39–5.52 U/g protein). A possible interpretation is that the liver-associated activity is more variable among species, whereas the intestinal mucosa sustains a relatively similar baseline level of chitinase activity. Because species identity was confounded with aquarium identity in the present design, however, this difference may also reflect aquarium-level differences in water chemistry, feeding response or microbial exposure, and it should be treated as a hypothesis for a replicated design to test.

Where between-species patterns are described in Section 3.2, they are attributed to the species concerned rather than to a dietary category. Each dietary category contained at most two species, each species was held in one aquarium, and the observed values of several species within a category varied more from one another than from those of species in other categories.

4.2. Tissue Distribution of Chitinase Gene Transcription and Its Relation to Enzyme Activity

Exploratory ΔCt profiles varied among tissues within several species, but the tissue ordering was strongly dependent on the normalisation procedure (Section 2.5). Because RNA input was not normalised to a common mass, amplification efficiencies were not determined and actin-family reference-transcript Ct values varied substantially among tissues, these profiles are treated as descriptive and hypothesis-generating only. Fish chitinases belong mainly to glycoside hydrolase family 18 [15,16].

The tissue with the lowest mean ΔCt differed among species (Section 3.3), but the relative ordering changed in seven of the eight species when the analysis was repeated using unnormalised target Ct values (Section 2.5, Table S7). These differences show that the apparent tissue ranking is sensitive to the normalisation approach. Because the actin-family reference transcript was not independently validated across tissues, RNA input was not normalised by mass, and amplification efficiency was not calibrated, the data do not support assigning a dominant tissue for chitinase transcription. The qPCR results are therefore retained as exploratory, within-species descriptions; no *p*-values or *q*-values are reported for this component.

The descriptive transcription profiles did not consistently parallel the tissue-associated enzyme activity profiles. Given the limitations described above, this lack of correspondence cannot be interpreted as evidence for or against a mechanistic relationship between transcription and enzyme activity. The qPCR component is used only to generate hypotheses for future validated analyses.

The qPCR results are therefore used only to describe within-species tissue distributions, and no between-species comparison of expression level is made. Most species were analysed

using species-specific primer sets; however, two primer sets were shared across species pairs (largemouth bass and black carp; bighead carp and silver carp; Table 3). No reference transcript containing both primer-binding sites was identified for the second species in either pair (black carp and silver carp). Because a single melting-curve peak indicates predominant amplification of one product but does not establish its sequence identity, and the amplicons were not sequenced, amplification of the intended target cannot be confirmed in these two species; their profiles are therefore retained as descriptive observations only. Three target accessions in the original primer records did not contain the corresponding primer sites and were replaced by the transcripts in which both sites occur (Table 3); this alters the annotation but not the primer sequences or the numerical qPCR data.

4.3. Gut Microbial Composition and Chitin-Related Functional Potential

The metagenomic component of this study must be interpreted in light of its data-availability profile (Section 3.5, Table S1). Ten of the twenty-three libraries had fewer than 50,000 classified reads after host-read removal and were dominated by fungal taxa that can occur in reagent-, soil- or plant-associated contexts. No library from common carp or black carp met the predefined threshold for downstream functional interpretation, whereas only one library each was retained for crucian carp and largemouth bass. The ten low-depth libraries were retained in the sequencing-depth statistics but excluded from downstream functional interpretation. This constraint shaped the scope of the microbial conclusions.

No test of a species effect on community structure was performed, because each species was held in a single aquarium and the libraries of a species are subsamples of one aquarium rather than independent replicates. In the ordination including all twenty-three libraries (Figure 4A), the ten low-depth libraries formed a separate depth-associated cluster irrespective of species. Among the thirteen retained libraries (Figure 4B), some within-species clustering was apparent for the four species represented by two or more retained libraries—silver carp, bighead carp and blunt snout bream with three each and grass carp with two; crucian carp and largemouth bass contributed one retained library each and common carp and black carp none, so no statement about within-species clustering is possible for them. This pattern is descriptive only and cannot be interpreted as a species effect.

With these caveats, the retained libraries contained annotations associated with chitin turnover. GH18 was annotated in every retained library, GH19 and GH20 in eleven of thirteen, and CBM5 in ten. The chitinase orthologue K01183 was detected in twelve libraries, while K13381 (bifunctional chitinase/lysozyme) was detected in three. These two KOs are associated with chitin hydrolysis. Other detected KOs, including K01207, K12373, K18676, nagA (K01443), nagB (K02564) and nagK (K00884), are associated with hexosaminidase-related processing or downstream amino-sugar metabolism. Their presence is consistent with predicted microbial functional potential but does not establish that the corresponding genes are expressed or that dietary chitin is actively degraded in vivo. Although several polysaccharide-associated taxa were detected, including members of Bacteroidota [45,46], taxonomic abundance was not linked directly to the chitin-related functional annotations; therefore, no specific taxon is assigned a chitinolytic role in this study.

These data are consistent with a gene repertoire for microbial chitin turnover and for downstream amino-sugar metabolism; they do not show that the repertoire is used. The KEGG orthologue assignments, which were obtained from the mappings distributed with HUMAnN3, carry the functional argument, whereas the CAZy profiles contribute supporting, exploratory evidence only (Section 2.7). Metagenomic annotation reports potential, not expression or flux. Demonstrating that the gut microbiota of these fishes actually degrades dietary chitin would require metatranscriptomic evidence, microbial enzyme assays, isolation of chitinolytic strains, or detection of chitin-derived metabolites

in the gut, none of which was performed here. The genera identified in these libraries also include taxa typical of water, sediment and laboratory reagents, so a contribution of environmental, dietary or reagent-associated DNA to these profiles could not be excluded. No usable metagenomic library was obtained for black carp or common carp, limiting the coverage of the chitin-focused comparison and motivating repeat sampling of these species.

4.4. Limitations and Future Directions

The findings of this study should be interpreted within several important limitations related to experimental design, biological variation, metagenomic data availability, analytical scope, and exploratory transcription profiling. These limitations mainly affect the strength of biological inference rather than the value of the dataset as a comparative descriptive baseline.

First, each species was maintained in a single aquarium, meaning that species identity was completely confounded with aquarium identity. The three composite samples obtained from each species therefore represent within-aquarium subsamples rather than independent species-level biological replicates. Consequently, the observed between-species patterns should be interpreted as descriptive observations rather than statistically supported species effects. Mean body weight also varied markedly among species (28.50 ± 2.48 to 246.55 ± 5.78 g), and the species differed in feeding ecology and likely nutritional requirements; these factors may influence digestive physiology and gut microbial composition but cannot be separated from species or aquarium effects in the present design. Future studies should incorporate independently replicated aquaria for each species and consider body size and feeding ecology in the sampling design.

Fish were acclimated for seven days and offered the same transitional diet to apparent satiation; however, this period was intended to reduce short-term handling-related variation rather than to eliminate the effects of previous diets or rearing conditions. Quantitative feed intake, species-specific feeding behaviour, detailed water-quality parameters, and the interval between the final feeding and sampling were not documented sufficiently for this dataset. These uncontrolled factors may have contributed to variation in tissue-associated enzyme activity and gut microbial composition and should be standardised or recorded in future replicated experiments.

Second, the metagenomic dataset was constrained by uneven library quality. Ten of twenty-three libraries contained insufficient classified microbial reads and were excluded from downstream functional interpretation, resulting in complete or partial loss of metagenomic representation for several species. In addition, the absence of extraction controls and detailed metadata for some sequencing procedures limited the ability to determine the contribution of non-target DNA sources. Future studies should include comprehensive quality-control procedures, replicated sampling and improved sequencing depth to provide more robust microbial functional comparisons. Among the retained libraries, AN3 had 236,818 classified reads, substantially fewer than most other retained libraries; low-abundance functional annotations may therefore have been less likely to be detected in this library.

Third, the transcriptional results should be considered exploratory. Because RNA input was not normalised by RNA mass, the reference gene was not independently validated across all tissues, and amplification efficiency was not determined, qPCR data were interpreted as descriptive transcription profiles rather than quantitative estimates of chitinase expression. Future studies should validate reference genes, confirm target amplicons and integrate transcriptomic or protein-level approaches to better resolve the relationship between chitinase transcription and enzymatic activity.

Finally, the present study measured tissue-associated chitinase activity and predicted metagenomic functional potential, but it did not directly evaluate dietary chitin utilisation. Controlled feeding experiments incorporating defined chitin-containing diets, apparent digestibility measurements, enzyme activity assays, and functional microbial validation are required to determine whether the observed patterns translate into physiological utilisation of dietary chitin.

5. Conclusions

Tissue-associated chitinase activity signals were detectable under the present assay conditions in all digestive tissues examined across the eight freshwater teleost species. The liver-associated sample had the highest observed tissue mean in seven species; however, none of the within-species tissue comparisons remained significant after correction for multiple testing. Of the 23 metagenomic libraries, 13 from six species met the operational classified-read threshold, with no libraries retained from common carp or black carp. The retained libraries contained annotations associated with chitin hydrolysis (K01183 and K13381) and downstream amino-sugar metabolism (including nagA, nagB and nagK). These annotations indicate predicted functional potential rather than demonstrated microbial chitin degradation or in vivo dietary chitin utilisation.

The exploratory qPCR profiles were not used as inferential evidence because of limitations in RNA input normalisation, reference-gene validation and amplicon identity verification. Furthermore, because each species was represented by a single aquarium, between-species patterns should be interpreted descriptively rather than as statistically established species effects. Future studies should use independently replicated aquaria, defined chitin-containing diets, validated molecular assays and direct measurements of chitin digestibility and degradation to determine whether the patterns observed here translate into physiological utilisation of dietary chitin.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fishes11110668/s1, Figure S1: Phylum-level composition of retained gut metagenomic libraries; Figure S2: Genus-level composition of retained gut metagenomic libraries; Figure S3: KEGG level-2 pathway categories in retained gut metagenomic libraries; Figure S4: Exploratory relative transcription profiles of chitinase genes within species ($2^{-\Delta\Delta Ct}$); Table S1: Sequencing quality-control statistics and data-retention characteristics of the metagenomic libraries, including retained/excluded status; Table S2: Library-level abundance (CPM) of chitin-related CAZy families in retained libraries; Table S3: Library-level abundance (CPM) of chitin-related KEGG orthologues in retained libraries; Table S4: Tissue-associated chitinase activity and within-species Friedman-test results; Table S5: Composite-level chitinase qPCR Ct and ΔCt values, with underlying technical replicate measurements; Table S6: Descriptive summary of chitin-related CAZy families and KEGG orthologues grouped by species in retained metagenomic libraries; Table S7: Tissue-associated variation in β-actin Ct values and descriptive sensitivity analysis based on unnormalised target Ct values; Table S8: Exploratory relative chitinase transcription estimates ($2^{-\Delta\Delta Ct}$) across tissues within species. No inferential statistics are reported for the exploratory qPCR component.

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