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Unraveling the Role of Bacteria in Nitrogen Cycling: Insights from Leaf Litter Decomposition in the Knyszyn Forest

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Abstract: Microorganisms are vital in leaf litter decomposition and contribute significantly to global nutrient cycling. However, there is a need for improved understanding of the taxonomic and functional diversity of litter-associated bacteria. The Knyszyn Forest comprises a unique ecosystem providing diverse microhabitats for microorganisms in central Europe, similar to the southwestern taiga in many respects. This study presents the results of high-throughput sequencing performed for *Betula pendula*, *B. pubescens*, and *Carpinus betulus* litter-associated microbial communities from northern Poland. Microbial assemblage composition and structure at different stages of litter decomposition revealed the domination of phyllosphere-associated taxa of *Sphingomonas* and *Pseudomonas* in bacterial communities in the early stages. Meanwhile, at the later stages of decomposition, the representation of soil-associated bacterial communities, such as *Pedobacter*, was higher. This study identifies key bacteria (*Pedobacter*, *Mucilaginibacter*, and *Luteibacter*) as pivotal in nutrient cycling through cellulose and hemicellulose decomposition, dominating later decomposition phases. Taxonomic analysis based on functional markers associated with nitrogen metabolism highlights the pivotal role of specific Pseudomonadota (Proteobacteria) taxa in driving nitrogen cycling dynamics during litter decomposition. Most of these taxa were unclassified at the genus level, particularly in the later stages of litter decomposition, and are crucial in mediating nitrogen transformation processes, underscoring their significance in ecosystem nutrient cycling.

Keywords: bacteria; nitrogen cycling; forest-forming trees; leaf litter decomposition

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1. Introduction

Leaf litter decomposition is a key process in the global nutrient cycle, driven primarily by microorganisms [1–3]. Among these microorganisms, fungi, and bacteria, with their ability to break down both labile compounds within substratum and chemically stable biopolymers, play central roles [1]. While past research has focused on fungal species composition, analyses of their changes during decomposition and their metabolite profiles often relied on culture-dependent studies [1,2] or on limited marker gene analysis and amplicon-sequencing methods [4]. The functional capacity of microbial communities, particularly bacteria in temperate forest ecosystems, is still understudied.

Temperate forests are relatively rich in nutrients. Terrestrial ecosystems have numerous vascular plant species with individual phytobiomes, which produce substrata of litter that are decomposed and release nutrients. [3]. The role of microorganisms in developing host plant resistance to pathogens, plant functioning, and energy transformation of dead plant material is often underestimated, especially in relatively species-rich temperate forests [2].

As others have noted, the functioning of terrestrial ecosystems is shaped by complex interactions involving plants, root-associated organisms, and decomposers, which influence both above- and below-ground processes [5]. In mixed species forest ecosystems,

individual trees impact decomposition directly through their species–specific litter quality and indirectly through modifications to micro-environmental conditions and soil biota composition [1–5].

Litter-decomposing microorganisms are diverse microbes that play pivotal roles in the breakdown and decomposition of plant litter in ecosystems. These microorganisms, including bacteria, fungi, and other microbial species, contribute to organic matter decomposition, releasing nutrients back into the soil and influencing nutrient cycling in terrestrial environments [6–9]. The complex interactions among these microorganisms shape the dynamics of litter decomposition, affecting ecosystem functioning and the overall health of natural habitats. Studying the composition and activities of litter-decomposing microorganisms provides valuable insights into the complex web of interactions that sustain ecological processes in forest ecosystems [10].

The Knyszyn Forest, located in northern Poland, comprises a unique habitat for plants and distinctive central European microhabitats for microorganisms due to many similarities to the southwestern taiga [11]. In northern Poland, researchers have paid particular attention to vascular plants and fungal species. At the same time, the bacterial communities associated with leaf litter and their role in nitrogen cycling remain underexplored, representing a critical knowledge gap.

To address these gaps, our study employs a comprehensive whole-metagenome analysis to unravel the genetic potential of leaf litter-associated microbial communities. While much attention has been directed towards fungal communities, this research focuses on the composition and structure of bacterial communities, given the scarcity of publicly available data on leaf litter decomposing bacteria.

Leaf litter-decomposing bacteria exhibit a unique synthesis of all twenty amino acids de novo, relying on complex metabolic pathways that utilize glucose as a carbon source and ammonium as a nitrogen provider. This distinct metabolic versatility underscores the importance of studying these bacteria to unravel the intricate mechanisms driving nutrient cycling and organic matter decomposition in forest ecosystems [10].

This study aims to examine the taxonomic composition of bacterial communities decomposing leaf litter and their role in nitrogen cycling in the Knyszyn forest ecosystem. We propose that specific bacterial genera exhibit heightened nitrogen cycling activity at different leaf litter decomposition stages within the Knyszyn Forest. Expanding upon the findings and observations obtained previously by other researchers [11,12], regarding the involvement of *Pedobacter* in cellulose and hemicellulose breakdown, we hypothesize their differential participation across distinct decomposition phases. Previous authors [12] have pointed out the necessity for a more comprehensive exploration of microbial nitrogen cycling contributions in forest ecosystems, highlighting a vital area for future research. Expecting fluctuations in abundance among these genera throughout decomposition stages, we posit their heightened involvement in nitrogen cycling processes as decomposition progresses. Based on their metabolic adaptability, we hypothesize that these bacterial taxa are pivotal in driving nitrogen transformation processes, significantly influencing nutrient dynamics in the Knyszyn Forest ecosystem.

This study focuses on taxonomic and functional diversity within bacterial communities, mainly emphasizing on functional diversity related to nitrogen assimilation and dissimilation genes.

2. Materials and Methods

2.1. Study Area, Experiment Design, and Litter Decomposition

The study was conducted in the Kopna Góra arboretum, situated in northern Poland within theKnyszyn Forest at coordinates 53°14′ N, 23°29′ E, located on flat glaciofluvial sand sediments at an elevation of 135 m above sea level. The dominant soil type is likely to be podzols [13]. These acidic soils are characteristic of forested areas with cool, humid climates, such as the Knyszyn Forest, and are commonly associated with coniferous forests. The area receives an average annual precipitation of 650 mm (1993–2007) and experiences

an average annual temperature of 6.9 °C, featuring a 200-day growing season. According to the data from the WorldClim database, the annual mean temperature in the Knyszyn Forest is 6.68 °C, with a range from 22.3 °C in the warmest month to −6.4 °C in the coldest month, while annual precipitation averages 587 mm, reaching its peak at 219 mm during the wettest quarter and decreasing to 90 mm in the driest quarter [14]. The ground is covered by moss, predominantly *Pleurozium schreberi* and *Dicranum polysetum*, along with cowberry (*Vaccinium vitis-idaea*).

For incubation, 10 g of litter from *Betula pendula* (silver birch), *B. pubescens* (downy birch), or *Carpinus betulus* (common hornbeam) was placed in 200 × 200 × 20 mm bags with a 1.5 mm mesh bottom, positioned on the existing leaf litter and moss beneath trees. Bags were covered with 5 mm mesh to prevent wind dispersal and the ingress of foreign materials. Leaf litter samples were collected four times: at the beginning of decomposition (after leaf fall, 0 months) and after 6, 15, and 18 months of decomposition. A detailed description of the field experiment and photos of sampling sites and litter were given in our previous study [15]. At the onset of our experiments, freshly fallen leaves from three distinct tree-forming plant species were collected into sterile Petri dishes (two plates at each litter type). Subsequently, these leaves underwent gradual decomposition through successive stages facilitated by microbial activity. Sampling activities were conducted across various seasons, including November 2020, May 2021, February 2022, and May 2022, facilitating the observation of seasonal changes. After sampling, the material was delivered to the laboratory and prepared for molecular analysis.

2.2. DNA Extraction, Sequencing, and Bioinformatics

Dried litter underwent homogenization with liquid nitrogen using sterile hand homogenizers to break down cell walls. DNA extraction utilized the E.Z.N.A.® Soil DNA Kit (Omega Bio-Tek, Norcross, GA, USA), with optimization based on previous work [15]. DNA extraction was performed 5–10 times per sample, depending on DNA amount and quality. Fresh leaf-litter samples, with a higher host DNA content, presented challenges. Whole-metagenome sequencing was conducted by Eurofins Genomics AT GmbH on the Illumina NovaSeq 6000 platform (NovaSeq 6000 S4 PE150 XP sequence mode, aiming for 20 M reads per sample).

Bioinformatic analysis occurred on the KBase platform following steps presented in the tutorial by Dylan Chivian Learn How KBase Can Help You | KBase. Paired-end reads underwent quality control with FASTQC [16,17] and were trimmed using Cutadapt [18]. The study employed an assembly-based approach using MEGAHIT [19,20]. Kaiju [21] was used to perform reads-based taxonomic classification. Bins were obtained using MetaBat2 [22], and the quality of bins was estimated using CheckM [23]. After that, bins were selected for further analysis, including the annotation and phylogenetic analysis of MAGs (metagenome assembled genomes). The dRep tool was used to de-replicate selected bins [24]. Reference databases were from the National Center for Biotechnology Information (NCBI) [25], and taxonomic assignments of MAGs were analyzed with DAS Tool [26] using the Genome Taxonomy Database (GTDB) [27]. Functional genome annotation was performed by applying Fama apps [28,29] and DRAM (Distilled and Refined Annotation of Metabolism) [30] within the KBase server. Statistical analysis was performed in R [31]. Raw reads were deposited under BioProject ID: PRJNA1001592.

3. Results

3.1. Taxonomic Composition and Structure of Bacterial Communities Using Kaiju

The percentage of sequences classified by RefSeq using the NCBI database varied from 19% to 60%. Pseudomonadota comprised more than 50% of total bacterial reads at all stages of leaf litter decomposition in all samples. Alphaproteobacteria dominated at the earliest stages of decomposition, with the highest percentage in the hornbeam-associated community (73% of total reads). The percentage of Alphaproteobacteria decreased in all samples after 15 months of litter decomposition. The next most abundant groups were

Actinomycetota (previously Actinobacteria) and Bacteroidota (previously Bacteroidetes), varying from 5 to 23% of the samples. At the beginning of decomposition of birch leaf litter, Bacillota (Firmicutes) was also abundant, comprising almost 8% of total reads (Figure 1).

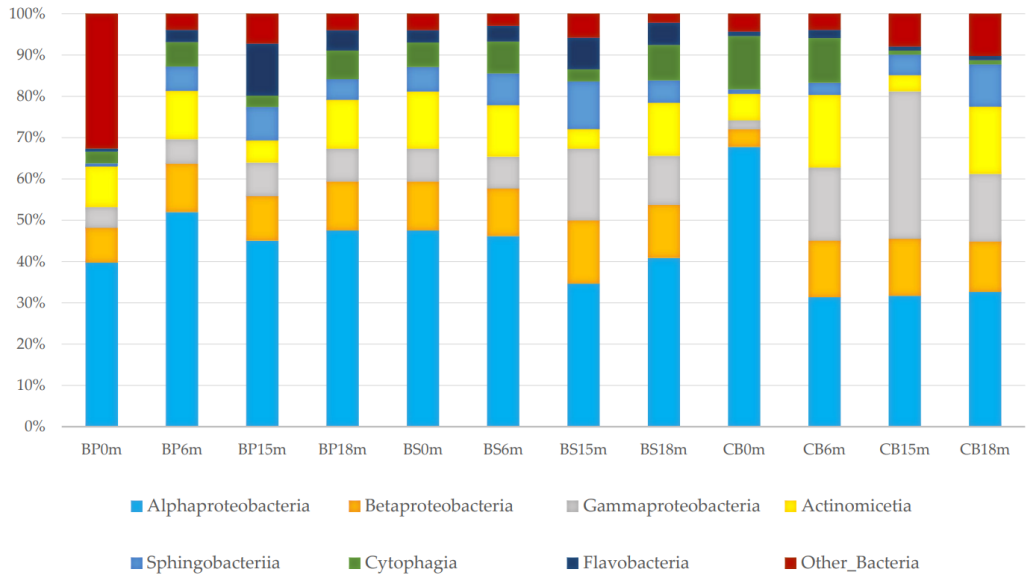

Figure 1. Taxonomic classification of bacterial reads at class level. Names of samples correspond to the species providing leaf litter (BP, *Betula pendula*; BS, *Betula pubescens*; CB, *Carpinus betulus*) and phase of decomposition (0 m, 6 m, 15 m, and 18 m are 0, 6, 15, and 18 months of decomposition, respectively).

In most samples, regardless of the stage of decomposition, the prevalent bacteria were *Sphingomonas* (4%–12% of classified reads in the sample [crs]), *Pseudomonas* (2%–14% [crs]), *Pedobacter* (1%–6% [crs]), *Flavobacterium* (1%–16% [crs]), and *Rhodococcus* (0.9%–12% [crs]). The percentage of Comamonadaceae (*Brukholderia*) was much higher in birch samples than in hornbeam. The percentage of *Rhodanobacter* was much higher in hornbeam samples, increasing 15 and 18 months after the start of decomposition (12 and 8%, respectively) (Figure 2).

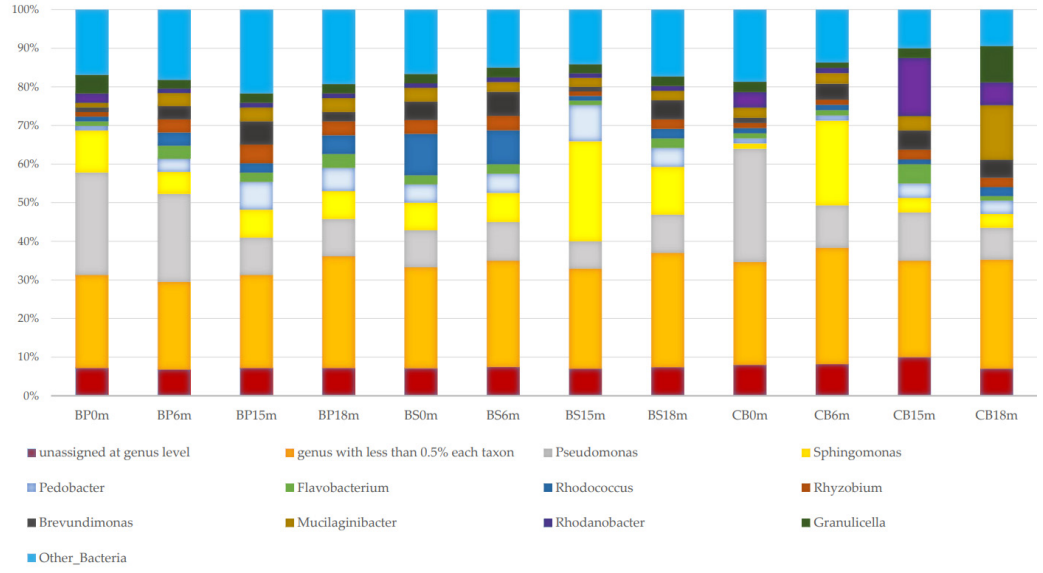

Figure 2. Taxonomic classification of bacterial reads at species level. Names of samples correspond to the species providing leaf litter (BP, *Betula pendula*; BS, *Betula pubescens*; CB, *Carpinus betulus*) and phase of decomposition (0 m, 6 m, 15 m, and 18 m are 0, 6, 15, and 18 months of decomposition, respectively).

3.2. Functional Groups Analysis

We analyzed a set of metagenomic reads from various incubation times (0 m, 6 m, 15 m, and 18 m) for silver and downy birch and hornbeam (BP, BS, CB) to investigate functional capacity of litter decomposing bacterial communities and variation in nitrogen metabolism capacity of microbial communities. Each dataset includes the total number of forward and reverse reads, mapped reads, predicted average insert size, and predicted average genome size. For instance, the BP-0 m dataset has 12,999,682 forward and reverse reads, with 4257 and 4222 mapped reads, an average insert size of 361, and an average predicted genome size of 12,858,168 (Table S1). These results provide important information regarding the nitrogen metabolism potential of microbial communities across different conditions and locations. Functional groups analysis conducted with the Fama tool indicates specific function categories, such as ammonification, denitrification, nitrate assimilatory and dissimilatory reduction, nitrite assimilation, nitrogen fixation, and urease metabolism. We observed shifts in the numbers of genes responsible for nitrogen fixation at different phases of litter decomposition; the highest number of nitrogen fixation genes was detected in downy birch metagenomes at the first two phases of decomposition. Based on clustering of the heatmap, we see that communities decomposing hornbeam leaves formed individual clades. Only one hornbeam-associated microbial community in the latest phase of decomposition (18 months) was grouped with birch microbial communities (Table S2).

Comparisons between birch and hornbeam litter samples at different stages of decomposition reveal both similarities and differences in the abundance and diversity of functional genes related to nitrogen metabolism. Increased genes associated with nitrate reduction are observed in the latest phase of birch decomposition for nitrate and nitrite reduction. *nar*G/nxrA remains significant, suggesting ongoing nitrate reduction. For hornbeam, there is also increased *nar*G/*nxr*A in the latest decomposition phase, indicating a potential shift towards nitrate reduction. Assimilatory nitrate reduction group analysis revealed that genes related to assimilatory nitrate reduction (*nas*A and *nas*B) are abundant in both birch and hornbeam. In the latest phase of birch and hornbeam litter decomposition, there are increased nitrite reductase genes (*nir*B and *nir*D), indicating enhanced nitrite reduction. Both birch and hornbeam samples show the presence of genes associated with nitrogen fixation. Genes involved in denitrification (*nir*K, *nir*S, *nir*U, *nos*Z) are detected in both birch and hornbeam samples, indicating the potential for denitrification processes. Urease genes (*ure*A, *ure*B, *ure*C) are abundant in bacteria decomposing both birch and hornbeam litter, demonstrating ongoing urea hydrolysis and ammonia release. Genes related to nitric oxide reduction (c*nor*C, c*nor*B, and qNOR) are present in birch and hornbeam, showing the possibility for nitric oxide metabolism. Both birch and hornbeam litter exhibit similarities in the types of nitrogen metabolism genes present, emphasizing common microbial pathways during decomposition. Differences in the abundance of specific genes indicate unique microbial community dynamics and metabolic activities in response to litter type and decomposition stage. These results demonstrate the complex and dynamic nature of nitrogen cycling during litter decomposition, with microbial communities adapting to changing conditions over time (Figure 3).

In the latest phase of hornbeam litter decomposition, there are increases in genes related to nitrate reduction, assimilatory nitrate reduction, and nitrite reduction. Nitrogen fixation, denitrification, urease activity, and nitric oxide reduction genes are detected throughout leaf decomposition. Differences in gene abundance may indicate variations in microbial community composition and metabolic activity at different stages of decomposition. Taxonomic analysis of functional genes, particularly *nar*G/*nxr*A later in decomposition, revealed the prevalence of Gamma and Betaproteobacteria, with the most abundant taxa being unclassified. In birch samples, Pectobacteriaceae dominated. Taxonomy based on *nir*A showed that Rizhobiales and Chthoniobacterales were prevalent. These observations underscore the nuanced dynamics of nitrogen cycling during litter decomposition, highlighting the importance of understanding both functional genes and taxonomic profiles, including *nar*G/*nxr*A and *nir*A, for a comprehensive insight into mi-

crobial ecosystems. Pseudomonadota (Proteobacteria), many of which were not classified, generally predominated across all nitrogen cycling processes. This emphasizes the ubiquitous presence and significance of Pseudomonadota (Proteobacteria) in various stages of nitrogen cycling during litter decomposition (Figure 4).

DRAM-based functional gene annotation comparison revealed that significant differences in bacterial communities were observed at the beginning of decomposition in birch and hornbeam leaf litter decomposing communities (Figure 5).

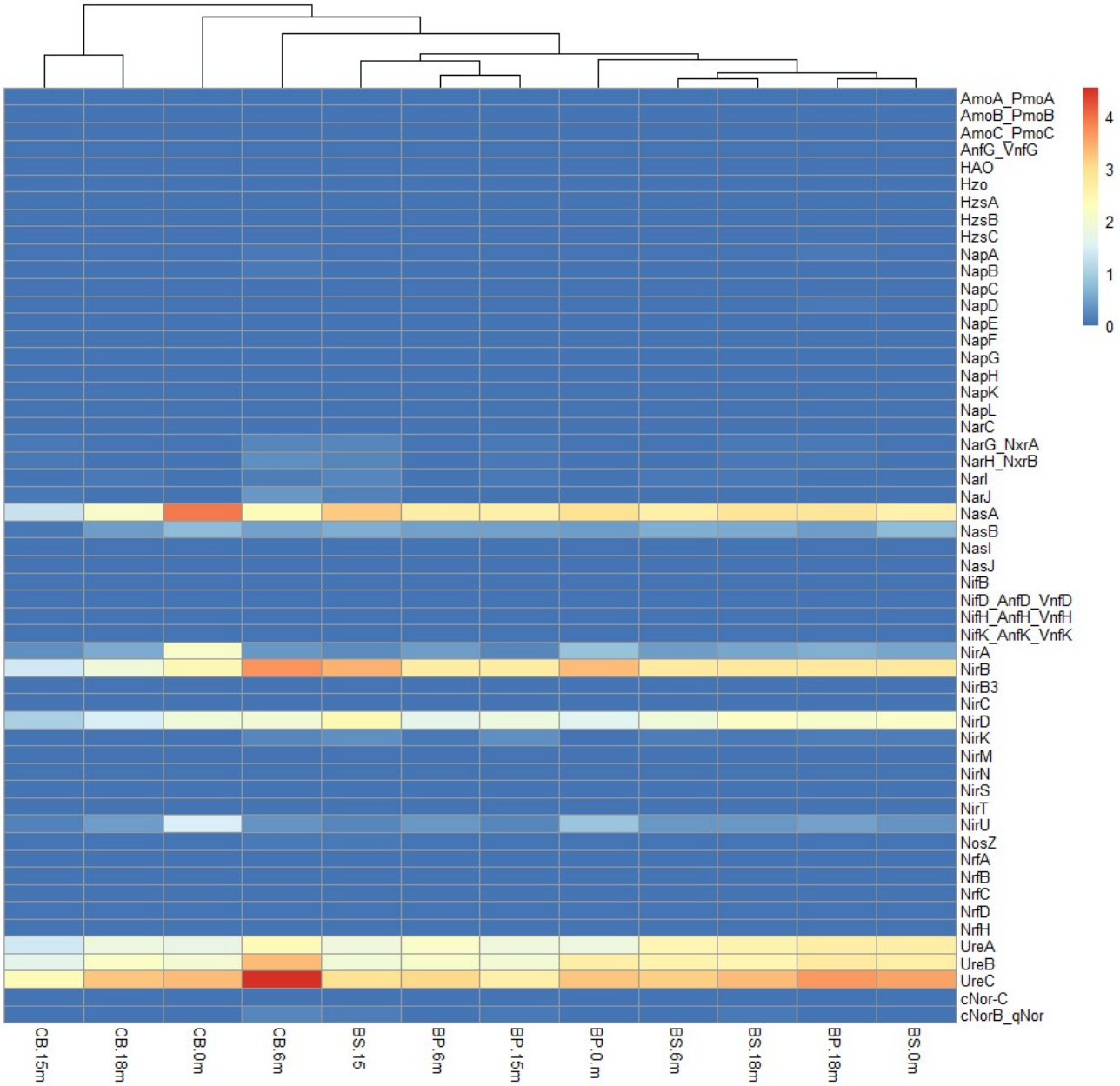

Figure 3. Heatmap of the functional profile of microbial communities. Names of samples correspond to the species providing leaf litter (BP, *Betula pendula*; BS, *Betula pubescens*; CB, *Carpinus betulus*) and phase of decomposition (0 m, 6 m, 15 m, and 18 m are 0, 6, 15, and 18 months of decomposition, respectively).

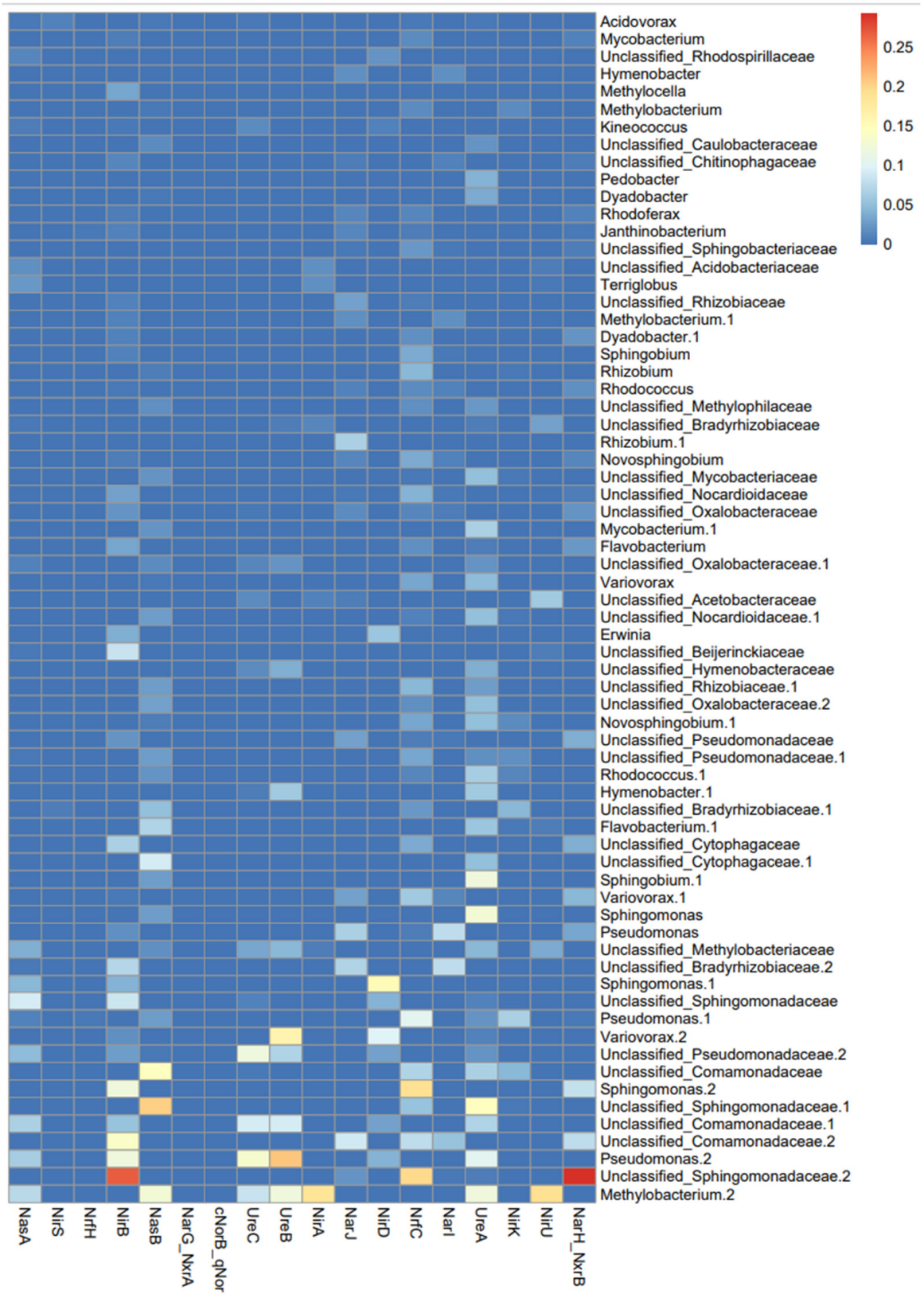

Figure 4. Heatmap of the co-occurrence of functional groups and bacterial taxa.

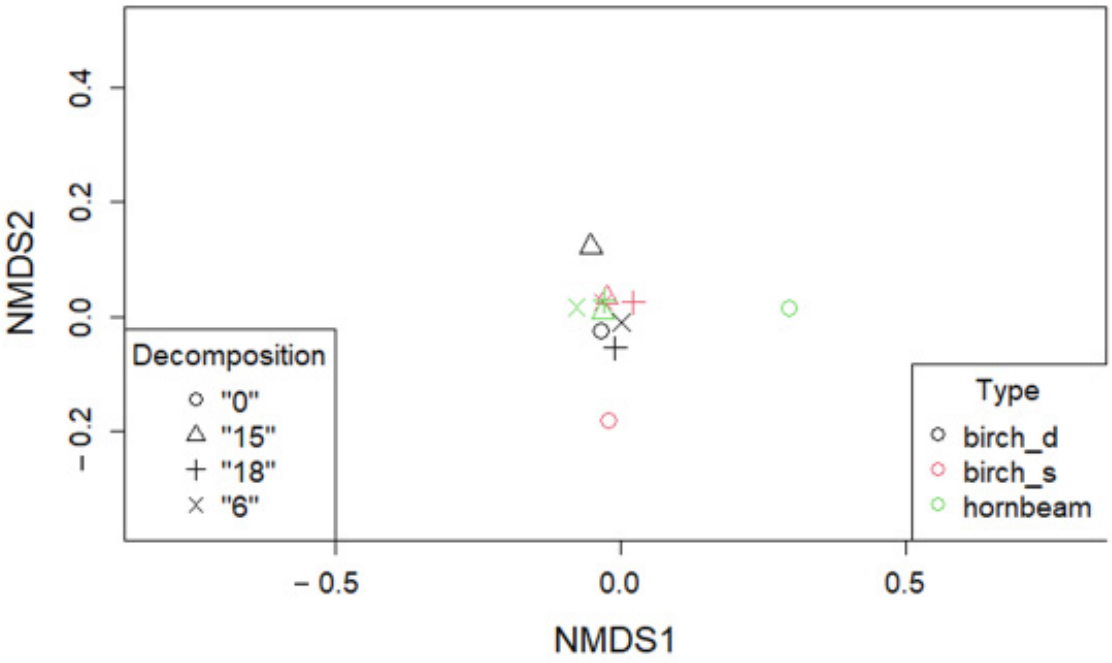

Figure 5. NMDS of the functional gene annotation of microbial communities (birch_s, *Betula pendula*; birch_d, *Betula pubescens*; hornbeam, *Carpinus betulus*). A stress value of 0.05 indicates an excellent fit for the NMDS ordination. The result of ANOSIM (Analysis of Similarities), the R statistic of 0.04, suggests that there is a moderate separation between the groups in your dissimilarity matrix.

The results of the bacterial functional genes annotation after 6, 15, and 18 months present high similarity of the communities, especially for birch_s (silver birch) and hornbeam.

The relationships among microbial communities during leaf litter decomposition were investigated using PERMANOVA (Permutational Multivariate Analysis of Variance) and ANOSIM (Analysis of Similarities). PERMANOVA revealed that differences between microbial communities at various decomposition stages lacked statistical significance ($p > 0.05$) despite a well-fitting NMDS ordination observed with a stress value of 0.05. Similarly, ANOSIM exhibited a moderate separation between groups (R = 0.04), yet this distinction did not reach statistical significance ($p > 0.05$). These outcomes suggest that although discernible differences exist in microbial communities, they may lack biological significance. Further exploration is essential to comprehend this ecosystem's underlying drivers of microbial dynamics.

3.3. Analysis of Bacterial MAGs (Metagenome-Assembled Genomes)

After optimization with the DAS Tool and quality control, we mapped the bins using GTDB. Bins that were >90% complete and <10% contaminated were selected for further analysis. The GTDB-based phylogenetic analysis revealed that bacteria belong to *Brevundimonas* (MAG of BS-15 m), *Granullicella* (CB-18 m), *Sphingomonas* (BP-6 m), *Telluria* (BS-0 m), *Flavobacterium* (BS-18 m), Solirubrobacteriaceae JAGIBJ01 (BP-18 m), *Rhodococcus* (BS-6 m), *Sphingomonas* N (CB-18 m), *Galbiataella* (CB-18 m), Caulobacter (BS-0 m), *Rhodanobacter* (CB-18 m), *Erwinia billingiae* (BS-15 m), *Pinirhizobacter* (CB-15 m), *Pedobacter* (BS-15 m), and *Rouxiella* (CB-6 m) clades. Most MAGs were not assigned to phylogenetically closely related species, often commonly characterized as closed species, as they fell outside its pre-defined ANI radius (File S1). The interactive Krona charts and statistics related to the tree are available as supplemental material. The best-quality leaf litter MAGs came from the silver, downy birches, and hornbeam at later phases of decomposition, whose corresponding ID is presented on the phylogenetic tree (Figure 6). However, leaf-litter-originated MAG analyses are challenging. Our results suggest that novel species of uncultivated bacteria can participate in critical biogeochemical processes.

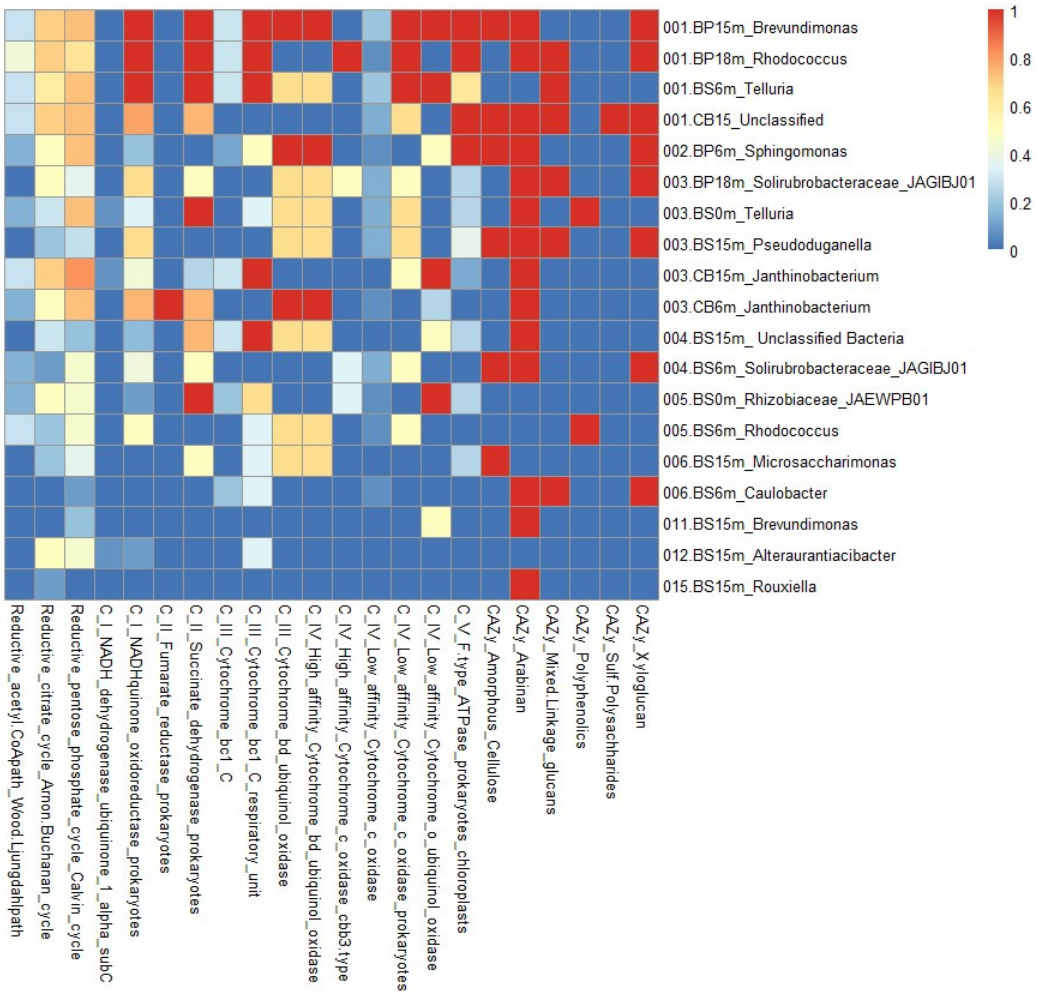

Figure 6. Functional annotation of selected MAGs.

Comparative analysis of the functional gene distribution within the obtained metagenome-assembled genomes highlights a notable trend: MAGs originating from birch litter decomposition communities demonstrate a greater functional capacity, with particularly pronounced differences observed at 15 months of decomposition (Figure 6 and File S1).

4. Discussion

The present study of leaf litter decomposing communities reveals taxonomic and functional shifts in microbial assemblages from the initial to the latter phase of decomposition. Notably, our investigation identifies changes over time in microbial communities, mainly dominated by phyllosphere-associated bacteria, especially in the case of hornbeam and birch during the initial stages of decomposition. The investigation into leaf litter decomposing communities reveals significant taxonomic and functional shifts in microbial assemblages from the initial to the latter phase of decomposition. Notably, we observed changes over time in microbial communities, particularly the dominance of plant-associated bacteria, especially in the case of hornbeam and birch during the initial stages of decomposition. However, it is worth noting that soil bacteria become more prominent later in decomposition, which may be influenced by factors such as litter type and decomposition stage [32,33].

The core community of leaf litter decomposing bacteria were represented by several genera, each with distinct ecological role. Functional gene analysis performed for genus *Sphingomonas* revealed its capacity for degrading organometallic compounds and have shown promise in enhancing plant growth under stressful conditions, such as drought, salin-

ity, and heavy metal exposure, attributed to the production of plant growth hormones [34]. *Rhodococcus*, notably abundant in downy birch litter, is well-known for stress-tolerant strains [35,36]. Genus *Methylobacterium*, dominant in hornbeam litter, has emerged as a crucial player in promoting plant growth and alleviating the impact of abiotic stresses in agriculture [37]. Our findings align with this, as we observed the presence of genes like *NifB*, *NifD_AnfD_VnfD*, and *NifH_AnfH_VnfH*, indicative of nitrogen fixation capabilities. This diverse group of Pseudomonadota (Proteobacteria) thrives in extreme conditions and enhances plant health, biomass, chlorophyll content, seed germination, and crop productivity through mechanisms like mineral solubilization, phytohormone production, nitrogen fixation, ACC deaminase activity, and the production of ammonia and siderophores [37,38].

Bacteria dominant in the latter phases of leaf decomposition, *Pedobacter*, *Mucilaginibacter*, and *Luteibacter*, might play a crucial role in the nutrient cycle by actively contributing to the decomposition of cellulose and hemicellulose in temperate oak forest ecosystems. MAGs analysis revealed that *Pedobacter* and *Mucilaginibacter* possess complex enzymatic systems with diverse carbohydrate-active enzymes, ensuring the efficient degradation of cellulose and hemicellulose. *Luteibacter*, on the other hand, utilizes a GH23 family protein for cellulose decomposition. This study underscores the structural diversity of enzymatic systems in cellulolytic soil bacteria, shedding light on the significant contributions of these bacterial taxa to the decomposition of plant polysaccharides and overall soil nutrient cycling [39,40]. Our analysis indicates the presence of genes associated with cellulose decomposition, such as amorphous cellulose and xyloglucan Carbohydrate-Active enzymes (CAZy), further supporting their functional role in nutrient cycling (Figures 4 and 6) [41].

Among the bacterial MAGs obtained within the present study were *Brevundimonas* [42] species, known for their versatility. They are found in various environments and are associated with nitrogen metabolism and the decomposition of organic matter. *Granullicella* species, also identified within MAGs of this study, are members of the Acidobacteriota (Acidobacteria) phylum. They are often involved in organic matter decomposition and nitrogen cycling in soil [43], which was confirmed in the present study based on the functional annotation (Figure 6). *Sphingomonas* species, the next most obtained MAGs, are known for their ability to degrade complex organic compounds [44]. The potential revealed by functional gene analysis confirms the capacity of *Sphingomonas* species to degrade the compounds. *Telluria* species might be associated with soil environments and could contribute to nutrient cycling processes [45], and further characterization is needed for specific insights into the nature of this genus. *Flavobacterium* species are common in aquatic environments and are involved in the degradation of complex organic compounds [46] and play an important role in nitrogen cycling (Figure 4). The present study revealed their potential involvement in nitrogen metabolism and assimilatory nitrate reduction (Figure 4). Solirubrobacteriaceae is a family within the phylum Actinomycetota (Actinobacteria). Members of this family may be involved in the decomposition of organic matter [44]. *Rhodococcus* [35,36] species are known for their metabolic diversity, and can degrade various compounds. They may be able to contribute to environmental processes like bioremediation. In general, *Sphingomonas* [47] species are associated with the degradation of complex organic compounds. Their presence might indicate involvement in nutrient cycling, particularly in nitrate reduction pathways (NasA) and potentially in urea hydrolysis (Figure 4). *Galbiataella* and *Caulobacter* are less well-studied, but previous studies suggest [47,48] an important role in microbial communities involved in nutrient cycling, and based on the present study, can be involved in urea hydrolysis and nitrate reduction (Figure 4). Contrary to the notion of complete denitrification by single microorganisms, specialist microbes performing specific nitrogen oxide reduction reactions are more common. Many microorganisms lack known genes for complete nitrate reduction, resembling incomplete denitrifiers. The complexity of microbial activities challenges the traditional classification of organisms based on classical nitrogen-cycling processes [49,50]. Our study revealed that analyzed microbial communities defy boundaries and adapt as needed in the continuous struggle for

survival. The evolving understanding of microbial functioning emphasizes the dynamic and versatile nature of nitrogen cycling [49].

Although the read-based taxonomic analysis showed the prevalence of particular taxa the analysis based on functional markers associated with nitrogen metabolism revealed the presence of many unclassified species of Pseudomonadota (Proteobacteria). This can be attributed to several factors: the investigated area is poorly studied, there is a lack of reference genomes in publicly available databases, and there might be novel taxa that play essential roles in nitrogen cycling in the Knyszyn Forest. These findings highlight the need for further studies to characterize these unclassified taxa better, expand reference databases, and improve our understanding of their functional roles in nitrogen-related pathways.

Utilizing the WorldClim database, we investigated the seasonal dynamics of bacterial communities in the Knyszyn Forest. The results highlight the influence of seasonal temperature and precipitation variations on bacterial populations. These fluctuations correspond to shifts in bacterial populations: *Pseudomonas* proliferates in autumn with moderate temperatures (~16.18 °C) and high precipitation (219 mm) but decreases in winter. *Sphingomonas* shows resilience to cold and dry conditions, thriving in winter. Spring and summer see a rise in microbial diversity, with *Rhodococcus* prevalent in moderate conditions. *Flavobacterium* and *Granulicella* maintain stable populations throughout the year.

This study investigates the taxonomic composition of bacterial communities engaged in leaf litter decomposition and their potential roles in nitrogen cycling within the Knyszyn forest ecosystem. Our hypothesis posits the identification of core microbial communities, characterized by functional gene analysis, implicated in various nitrogen cycling processes. Furthermore, the presence of unclassified taxa at the genus level suggests potentially novel taxa that may play significant roles in nitrogen cycling, yet to be described in the literature. Developing previous research highlighting the involvement of specific bacterial genera, such as *Pedobacter*, in cellulose and hemicellulose breakdown [10,11], we propose their differential participation across distinct decomposition phases. Our study underscores the impact of seasonal climate variations on microbial communities in the Knyszyn Forest. Understanding these dynamics is crucial for predicting ecosystem responses to climate change and in developing mitigation strategies. Further research is needed to explore ecological implications and microbial roles in ecosystem stability.

5. Conclusions

Microbial communities in the Knyszyn forest litter undertake dynamic shifts during litter decomposition, influenced by environmental conditions and tree traits. Shifts in microbial communities, from the initial phyllosphere-associated dominance to later stages led by soil bacteria, show dynamic changes that occur during leaf decomposition, with variations in gene abundance indicating unique patterns of bacterial types. Microbial nitrogen cycling in birch and hornbeam demonstrate commonalities in nitrogen metabolism genes but highlight dynamic nitrogen cycling through differences in gene abundance. Expanding on prior research, our observations of varying participation of specific genera like *Pedobacter* across different decomposition phases confirm our hypothesis. The functional marker analysis revealed many unclassified species of Pseudomonadota, likely due to the region's poorly studied microbial diversity, the lack of reference genomes in public databases, and the potential presence of novel taxa significant to nitrogen cycling in the Knyszyn Forest. The temperature and seasonal preferences observed among bacterial populations highlight their adaptive strategies to thrive in diverse environmental conditions. This study enhances our understanding of microbial dynamics during leaf litter decomposition, indicating the importance of taxonomic and functional aspects to obtain a comprehensive view of ecosystem interactions and further studies to determine the actual role of Bacteria in nitrogen cycling in the Knyszyn Forest.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/f15061065/s1. The following supporting information can be downloaded at: Unraveling the Role of Bacteria in Nitrogen Cycling: Insights from Leaf Litter Decomposition in the Knyszyn Forest Ecosystem—Google Drive, File S1: gtdbtk.backbone.bac120.classify-ITOL; File S2: krona_chart; File S3: Fama report; Table S1: Functional_profiles_combined; Table S2: Function_taxonomy_profiles_combined.

Author Contributions: Conceptualization, V.A.I. and N.K.; methodology, N.K.; software, N.K.; validation, N.K. and V.A.I.; investigation, V.A.I. and N.K.; resources, V.A.I.; data curation, N.K.; writing—original draft preparation, N.K.; writing—review and editing, I.J.; supervision, V.A.I.; project administration, V.A.I.; funding acquisition, V.A.I. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflicts of interest.

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