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Microbial Community Structure, Metabolic Function, and Phenotypic Characteristics of Sediment in Deep Coal Mine Underground Environment, China

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Abstract: Long-term coal mining has created unique microbial communities in deep coal mines. Revealing the microbial community structure and metabolic functions in the underground environment can contribute to a better understanding of the coal mine ecosystem. In this study, we collected underground sediment samples from producing mines in eastern China at mining depths of −400 to −1100 m and performed high-throughput sequencing. Results showed that most of the genera in the underground sediment can degrade organic matter, such as polycyclic aromatic hydrocarbons (PAHs), benzene, toluene, and xylene, etc. The dominant genera in the underground sediment were *Hydrogenophaga*, *Thauera*, *Pseudomonas*, *Rhodobacter*, and *Dietzia*. Samples were divided into coal roadway (CR) and rock roadway (RR) groups according to the sampling location. The microbial community structure differed significantly ($p < 0.05$) between these two groups of samples, with the distribution of main genera in the CR group samples showing a negative correlation with Cu and a positive correlation with temperature. The samples from the CR and RR groups were significantly different ($p < 0.05$) in their metabolic functions, including membrane transport, metabolism of other amino acids, folding, sorting, and degradation. Microorganisms in the RR group samples showed high resistance to heavy metals, while microorganisms in the CR group had higher degradation functions of organic pollutants. Bugbase phenotypic predictions indicated a high potential pathogenicity of microorganisms in coal mine sediment, which was mainly contributed by the genera *Hydrogenophaga*, *Pseudomonas*, *Geothermobacter*, and *Methylophaga*, etc. This study deepens the understanding of microbial communities in deep coal mine environments; however, the organic contamination and biological health risks of underground environments require extensive attention.

Keywords: deep coal mine; microbial community; metabolic functions; Tax4Fun; Bugbase

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1. Introduction

Coal plays an important role in China's energy system and approximately 90% of China's coal mines are underground mines [1]. Significant differences of temperature, humidity, wind speed, and nutrients exist between the underground and surface environments [2]. Under the combined effect of ground temperature and mine ventilation, the temperature inside the coal mine is usually stable within the range of 15–30 °C. Such environmental conditions are conducive to the growth and reproduction of various microorganisms. Measures such as ventilation and drainage required for mining activities can affect the distribution and succession of underground microbial communities; substances introduced by underground equipment, materials, and human activities, such as emulsions and motor oils [3], can also stimulate the proliferation and mutation of microorganisms. As a result, unique microbial communities can develop in the underground coal mine environment [4].

The microorganisms found in the underground mine environment may come from the geological microorganism of the coal seams, which play a significant role in the production of biogenic coalbed methane [5]. These microbes mainly include archaea such as *Methanobacterium*, *Methanomicrobium*, and *Methanolobus* and bacteria such as *Clostridia*, *Petrimonas*, *Actinomycetales*, and *Rubrobacterales*, etc. [6]. In our previous study, *Lactococcus*, *Pseudomonas*, *Mycobacterium*, and *Bacillus* were discovered to be the dominant strains in the underground environment of coal mines at the −700 m level [7]. Microorganisms play an important role in the transforming and cycling of substances and elements. For example, acid mine drainage is usually formed from pyrite in coal formations by the combined interaction of oxygen, water, and microorganisms (e.g., *A. ferrooxidans*) [8]. The degradation of some organic pollutants in the coal mine environment, such as polycyclic aromatic hydrocarbons and phenols, is also largely dependent on microorganisms [9]. During the mining process in coal mines, methane gas is a major threat to underground safety; its formation is related to the activity of microorganisms associated with methane metabolic functions [10]. In addition, several potential pathogens exist in the underground environment, such as *Klebsiella pneumoniae* and *Staphylococcus aureus* [11]. These pathogenic microorganisms and microbial metabolites, including bacterial toxins and fungal toxins, can endanger the health of underground workers. Currently, many studies have focused on the pollution, environmental impacts, and treatment techniques of the surface environment in coal mining areas; less attention has been paid to the underground environment. There is a lack of understanding of the characteristics and metabolic functions of microbial communities in the underground environment of deep coal mines.

Due to the limitations of sampling difficulties and analytical methods, few studies have reported on the microbial communities in the unique environmental conditions of underground coal mines. With the development of molecular biotechnology and the increasing research in environmental microbiology, Illumina-Miseq sequencing, for example, can directly and accurately detect microbial taxa in areas of low species abundance [12]. In this study, we collected underground sediment samples from producing mines in eastern China at mining depths of −400 to −1000 m and performed high-throughput sequencing. The objectives of this study were to (1) characterize the microbial communities and distribution of underground coal mines, (2) determine the effects of environmental factors on microbial communities, and (3) explore the specific metabolic functions of microbes in underground coal mines.

2. Material and Methods

2.1. Study Area

In this study, underground sediment samples were collected from Xuzhuang Coal Mine (XZ Mine) and Xinjvlong Coal Mine (XJL Mine) in the coal mining area of eastern China, respectively. XZ Mine is located in the Huanghuai alluvial plain area and is underlain by Quaternary strata [13]. The coal-bearing strata mainly include the Permian Taiyuan Formation, the Shanxi Formation, and the Lower Shihezi Formation, with a total thickness of 478.32 m, containing 20 coal seams with an average total coal seam thickness of approximately 13.69 m [13]. Coal mining in this mine started in 1985 and is divided into two mining levels: −400 m and −750 m. At present, the coal is mainly mined at the −750 m level. XJL Mine belongs to the North China Type Carboniferous-Permian Coal Field, with the middle and lower Ordovician series as the basement and the Neogene and Quaternary series as the overlying strata [14]. The main coal-bearing strata are the Taiyuan Formation and the Shanxi Formation, with a total thickness of 236.89 m and 24 coal seams. The average total thickness of the coal seams is about 17.79 m. Coal mining at this mine started in 2008 and the mine has two mining levels: −810 m and −950 m. Currently, mining activities are mainly carried out at the −810 m level.

2.2. Samples Collection

In January 2022, nine sediment samples (D1~D9) were collected underground at the depth of −400 m~900 m in XZ Mine, and six sediment samples (X1~X6) were collected underground at the depth of −700 m~1000 m in XJL Mine (Figure 1). Coal mining has been operated for almost 40 years at the XZ Mine and about 20 years at the XJL mine. The sampling locations and descriptions of sediment samples are shown in Table 1. In order to ensure the safety of the underground workers, the coal mining environment is usually under negative pressure ventilation, especially in the vicinity of the working face of the coal roadway. Most underground activities are carried out close to the coal roadway or workface, with the rock roadway being used mainly for hauling personnel and materials.

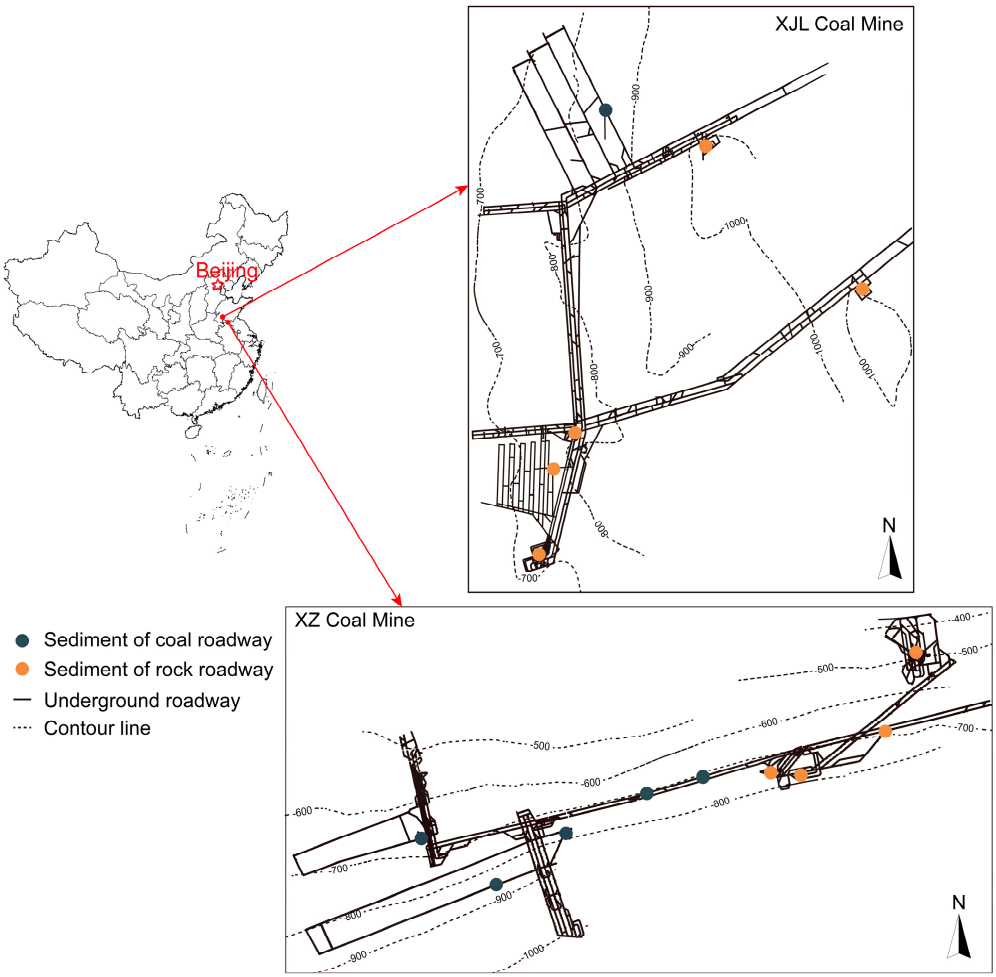

Figure 1. Study area and sampling sites.

Among all the sediment samples, six were collected from coal mining roadways or coal sludge deposition areas, while the others from rock roadways such as underground water tanks, drainage ditches, and rock roadways. During field collection, four subsamples were collected simultaneously near the sampling point and homogeneously mixed as representative samples for that point. After the removal of gravel and other lumps, the samples were mixed evenly and placed in sterile sampling bags. Upon completion of the underground sampling, the samples were placed in a 4 °C refrigerator and quickly transported back to the laboratory, where they were stored at −80 °C for microbial high throughput sequencing analysis.

Table 1. Locations and description of sediment samples from underground coal mines.

Number	Description	Coal Mine	Collected Location
D1	Sediment near the water tank	XZ	RR
D2	Rock roadway	XZ	RR
D3	Sediment near the water tank	XZ	RR
D4	Drainage ditch	XZ	RR
D5	Coal sediment	XZ	CR
D6	Coal sediment	XZ	CR
D7	Sediment near the excavation head	XZ	CR
D8	Working face	XZ	CR
D9	Working face	XZ	CR
X1	Sediment near the water tank	XJL	RR
X2	Drainage ditch	XJL	RR
X3	Working face	XJL	CR
X4	Drainage ditch	XJL	RR
X5	Drainage ditch	XJL	RR
X6	Drainage ditch	XJL	RR

Note: XZ—Xuzhuang Coal Mine, XJL—Xinjvlong Coal Mine, RR—rock roadway sediment samples, CR—coal roadway sediment samples.

2.3. Measurement of Physicochemical Parameters

Sediment samples were freeze dried in a freeze dryer (FD-1A-50, BioCool, Beijing, China). The pH of the solid samples was determined using the method described by Chen et al. [15]. The organic matter (OM) of the solid samples was determined by the potassium dichromate oxidation volumetric method [16]. Kjeldahl distillation was used to determine total nitrogen (TN) of samples. The total phosphorus (TP) content of the samples was measured by the sodium hydroxide molybdenum antimony anti-colorimetric method. Samples were analyzed according to the standard LY/T 1255-1999 for total sulfur (TS). A total of 0.2 g of dry sample, sieved through a 0.15 mm mesh, was digested in a microwave digestion bath. Then, the heavy metals Fe, Mn, Cu, and Zn were determined by inductively coupled plasma mass spectrometry (ICP-MS, TFS, Waltham, MA, USA) [17]. After sample digestion, the samples were analyzed for As by atomic fluorescence spectrometry (AFS8130, Titan Instruments, Beijing, China) [18]. All measurements were performed in triplicate.

2.4. High-Throughput Sequencing Process

The E.Z.N.A.® Soil DNA Kit (Omega Bio-tek, Norcross, GA, USA) was used to extract DNA from 1.0 g of solid sample. The concentration and quality of the extracted DNA were assessed using a Nanodrop® ND-2000 UV-Vis spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Amplification libraries were generated using modified prokaryotic universal primers 515F (5′-GTGYCAGCMGCCGCGGTAA) [19] and 806R (5′-GGACTACNVGGGTWTCTAAT) [20] for the V4 region of the 16S rRNA gene. A 2% agarose gel was used to extract the PCR products, which were further purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) and quantified using QuantiFluor™-ST (Promega, Madison, WI, USA). Purified amplicons (300 bp paired) were sequenced on the Illumina MiSeq platform (Illumina, San Diego, CA, USA) of Majorbio Bio-Pharm Technology Co. at Majorbio Bio-Pharm Technology Co. (Shanghai, China). Sequences were manipulated after processing on the Illumina MiSeq platform using the QIIME package (v 1.9.1) package [21]. Raw fastq files were demultiplexed, quality checked by Trimmomatic filtering, and merged using FLASH (v 1.2.11) [22]. Operational taxonomic units (OTUs) were clustered using the RDP classifier algorithm (v 2.11) based on the SILVA database, with a similarity control of 97% [23].

2.5. Statistical Analysis

The diversity of the microbial communities of the sediment samples was analyzed using the alpha diversity indices Sobs (number of OTUs), Ace, and Shannon [24]. Non-

metric multidimensional scaling analysis (NMDS) was used to analyze the similarities and differences in microbial communities between groups. Redundancy analysis (RDA) was used to determine the influence of physicochemical parameters on the microbial community. Microbial groups with significant differences between groups were identified by R-language based MetagenomeSeq difference analysis [25]. Tax4Fun (v 0.3.1) was applied to profile the potential functions of the samples' microbial community [26]. The Wilcoxon rank sum test was performed to determine the significance of differences between groups. The phenotype of the sample microbiome was predicted using the BugBase analysis tool (https://bugbase.cs.umn.edu/) [27]. The above data were performed on the Majorbio Cloud Platform (www.majorbio.com). Ballon plot, heat map, and violin plot were plotted by https://www.bioinformatics.com.cn (an online data analysis and visualization platform).

3. Results

3.1. Microbial Diversity of Underground Sediment

Fifteen libraries were generated from Illumina Miseq sequencing of the 16S rRNA genes, with a total of 1,094,451 effective reads; an average read length of reads was 253 bp. All libraries were rarified to the same depth (34,379/library) for further analysis. Based on the 97% similarity threshold, all sequences were finally clustered into 8276 operational taxonomic units (OTUs), including 8024 bacteria and 252 archaea. The number of OTUs belonging to the bacteria in the samples ranged from 810 to 3368, while the number of archaeal OTUs varied from 4 to 109. Alpha diversity indices, including Sobs (number of OTUs), Ace, and Shannon, were used to assess the microbial diversity of the underground sediment samples. XZ and XJL represented samples collected from Xuzhou Coal Mine and XinJvlong Coal Mine, respectively. In addition, samples collected from coal roadways were categorized as CR group, while those from rock roadways were categorized as RR group, based on underground sampling location. The results of the alpha diversity difference test showed that there was no significant difference in the number of OTUs, Ace, or Shannon indices of the underground sediment samples from the two coal mines (Figure 2a), while the Shannon indices of the sediment from the XZ Mine were slightly higher than those from the XJL Mine. The number of OTUs, Ace, and Shannon indices were higher in the RR group than in the CR sediment (Figure 2c). This was consistent with the results of the study of microorganisms in the underground sediments of the Quantai Coal Mine, which found that the microbial diversity of rock roadways was higher than that of the coal roadways [7]. Statistical analysis showed a significant difference in the Ace index between the two sample groups ($p < 0.05$), indicating that the relative abundance of microorganisms was significantly higher in the RR than in the CR. Beta diversity was used to compare the differences in microbial community structure between the groups. Results showed no significant differences between the microbial communities of the XZ and XJL mine sediment (Figure 2b), suggesting similarities in microbial community structure between the different underground coal mine environments. However, statistical results revealed significant differences ($p < 0.05$) in the microbial community structure between the CR and RR samples (Figure 2d).

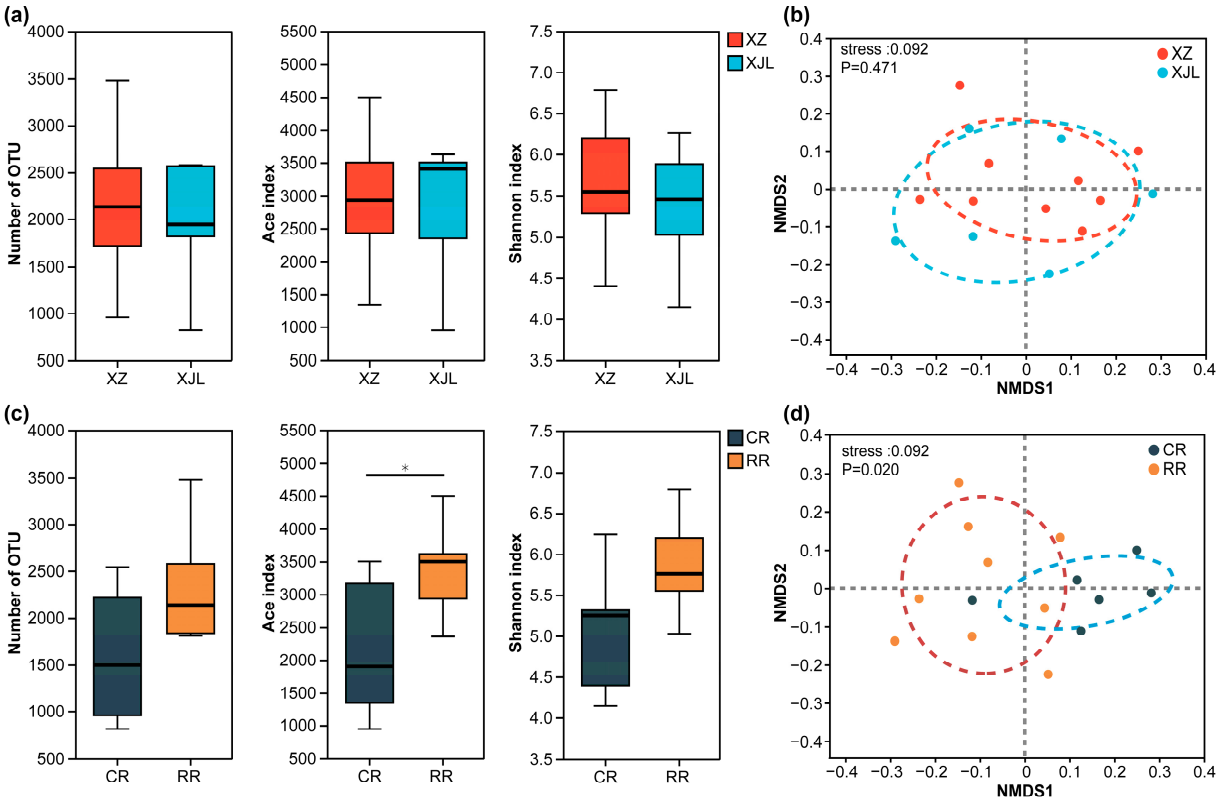

Figure 2. Comparison of alpha diversity indices of sediment from XZ and XJL mines (**a**). Beta diversity analysis of sediment from XZ and XJL mines (**b**). Comparison of alpha diversity indices of CR and RR group samples (**c**). Beta diversity indices of CR and RR group samples (**d**). (* $p < 0.05$)

3.2. Taxonomic Composition of Microbial Communities of Samples

In total, 77 known and candidate phyla were obtained from the 15 sediment libraries, of which 68 belonged to the bacteria and the others to archaea. The dominant phyla in the CR and RR samples are shown in Figure 3a. They mainly included *Proteobacteria*, *Actinobacteriota*, *Desulfobacterota*, *Chloroflexi*, *Firmicutes*, *Bacteroidota*, *Acidobacteriota*, *Planctomycetota*, and *Nitrospirota*. *Proteobacteria*, *Actinobacteriota,* and *Firmicutes* were also regarded as the dominant phyla in Quantai coal mine sediment [7]. Among them, the relative abundance of *Proteobacteria* and *Desulfobacterota* was higher in the samples of the CR group than in those of the RR group, while the relative abundance of the other dominant phyla was higher in the RR group samples. Comparison of the differences in the dominant phyla in the CR and RR samples by means of Metagenomeseq difference analysis revealed significant differences in the distribution of *Proteobacteria*, *Chloroflexi,* and *Planctomycetota* between the two groups ($p < 0.05$). *Proteobacteria*, with an average percentage of 67.28% and 44.61% in the CR and RR groups, respectively, was the most dominant phylum of samples. *Proteobacteria* in the sediment were mainly composed of *Alphaproteobacteria* and *Gammaproteobacteria*; their relative abundances in the CR samples were 52.15% and 15.12%, respectively, while in the RR they were 32.17% and 12.43%, respectively. In contrast, the relative abundances of *Chloroflexi* and *Planctomycetota* were higher in the RR samples, with average relative abundances of 7.25% and 3.36%, respectively, compared with 3.24% and 1.37%, respectively, in the CR samples. A total of nine archaea phyla were obtained from the underground environment samples and their relative abundance in the CR and RR samples is shown in Figure 3b. Statistical analysis showed that the significantly different archaeal phyla between the two groups were *Crenarchaeota*, *Nanoarchaeota,* and *Halobacterota* ($p < 0.05$). The average relative abundances of these archaea in the RR samples were 0.85%, 0.77%, and 0.49% respectively, compared with 0.29%, 0.06%, and 0.02% in the CR samples. The distribution of the major genera in the underground sediment samples is

shown in Figure 3c. *Hydrogenophaga*, *Thauera*, *Rhodobacter*, *Pseudomonas*, and *Marinobacter* were the dominant genera in the CR samples, with an average relative abundance of 7.65%, 5.53%, 3.69%, 3.13%, and 2.31%, respectively. The dominant genera in the RR samples were *Hydrogenophaga*, *Dietzia*, *Thiobacillus*, and *Nitrospira*, with average relative abundances of 2.58%, 2.26%, 2.14%, and 1.76%, respectively. Other studies of underground coal mine sediments have found *Pseudomonas* to be the dominant genus [7], which is consistent with our findings.

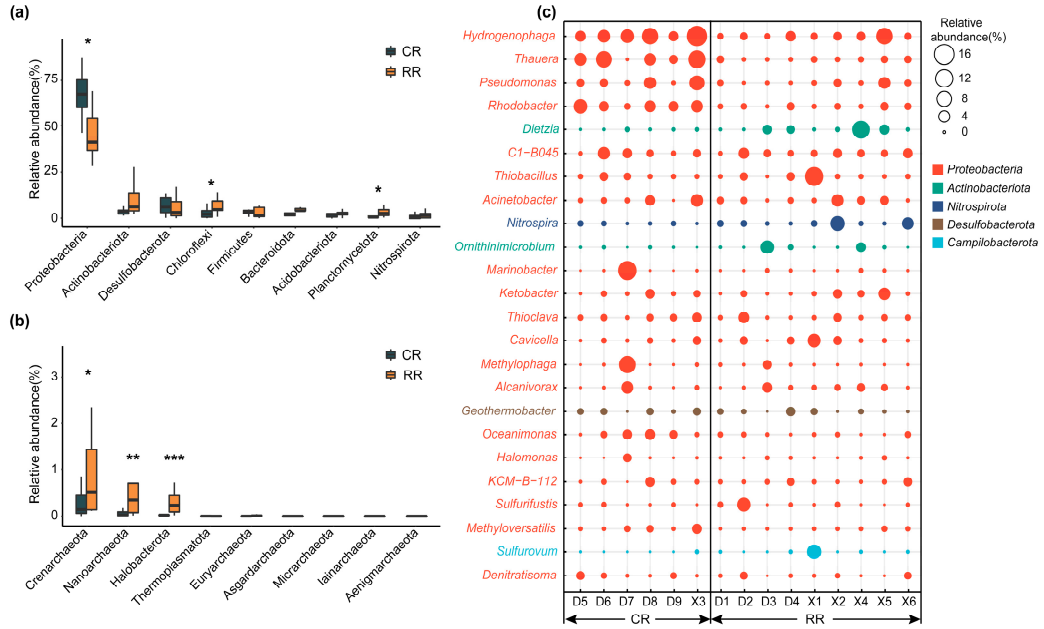

Figure 3. Comparison of the relative abundance of the main phyla in underground coal mine sediment (**a**) bacteria and (**b**) archaea. Distribution of the main genera (relative abundance > 0.5%) in sediment samples (**c**). (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

3.3. Effect of Physicochemical Parameters on Microbial Communities

The physicochemical parameters of underground sediment samples collected from deep coal mines are illustrated in Table 2. The temperature in the underground coal mine environment ranged from 15 to 30 °C and the average temperature was higher in the coal roadway than in the rock roadway. Except for the samples collected near the water reservoir at the −750 m level in the XZ Mine, the temperature of all the other samples exceeded 20 °C; such temperature conditions are suitable for the growth of most microorganisms. The pH range of the CR sediment ranged from 7.23 to 7.76 and was neutral overall, while the RR samples had a pH range of 7.55 to 9.24 and were neutral or weakly alkaline. The range of TN, TP, and OM contents in the RR sediment were 0.360–1.352 mg/g, 0.049–0.194 mg/g, and 0.073–1.384 mg/g, respectively, with mean contents of 0.827, 0.091, and 0.823 mg/L, which were slightly higher than those of the CR samples. However, the TS content in the sediment of the CR (average 14.417 mg/g) was higher than that of the RR group (8.667 mg/g). The heavy metals Mn, Fe, Cu, and As ranged from 0.031 to 1.831 mg/g, 4.855 to 116.200 mg/g, 0.023 to 0.070 mg/g, and 0.005 to 0.098 mg/g, respectively; the average content of these metals in the RR samples was higher than that in the CR samples.

Microbial community structure and diversity are strongly influenced by environmental factors [28]. RDA analysis results showed that the distribution of microorganisms in the sediment samples was significantly influenced by TS ($R^2 = 0.721$, $p < 0.05$) (Figure 4). Except for D7, D2, and D3, the other samples were distributed on the positive half-axis of the RDA2 axis, opposite to the direction of the TS, indicating that the distribution of microbial communities in the sediment was negatively correlated with the concentration of TS. Samples of RR were mainly distributed in the negative half-axis of RDA1, with acute angles with TN, Cu, Fe, As, Mn, and pH, indicating that the distribution of microbial

communities in the RR sediment was positively correlated with these physical parameters. The CR samples were predominantly distributed in the positive half-axis of the RDA1 axis, indicating that the microorganisms in the CR samples were positively correlated with the temperature, OM, TP, and Zn. Coal mining can generate heat during the mining process, resulting in higher temperatures in the coal roadway environment than in the rock roadway, which may explain the positive correlation between the microbial community and the temperature of the CR samples.

Table 2. Physicochemical parameters of underground sediment samples.

Number	Unit	Coal Roadway Sediment (CR Group)			Rock Roadway Sediment (RR Group)		
		Min	Max	Average	Min	Max	Average
Temp	°C	22.000	30.000	26.300	15.000	30.000	23.167
pH	—	7.230	7.760	—	7.550	9.240	—
TN	mg/g	0.312	0.837	0.561	0.360	1.352	0.827
TP	mg/g	0.066	0.135	0.088	0.049	0.194	0.091
OM	mg/g	0.005	1.165	0.486	0.073	1.384	0.823
TS	mg/g	5.900	50.000	14.417	2.900	28.000	8.667
Mn	mg/g	0.131	0.665	0.322	0.031	1.831	0.711
Fe	mg/g	9.190	47.240	24.865	4.855	116.200	57.838
Cu	mg/g	0.023	0.041	0.031	0.028	0.070	0.044
Zn	mg/g	0.061	1.022	0.241	0.012	0.350	0.173
As	mg/g	0.005	0.038	0.017	0.007	0.098	0.032

Note: TN—total nitrogen, TP—total phosphate, OM—organic matter, TS—total sulfur.

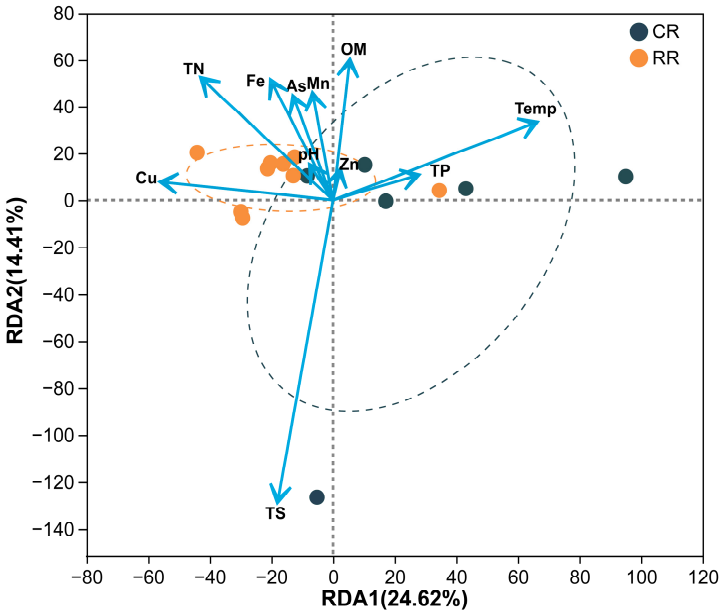

Figure 4. RDA analysis reveals the relationship between microbial communities and environmental factors in underground coal mine sediment.

4. Discussions

4.1. Characterization of Microbial Communities in Underground Coal Mine Sediments

The dominant genera in underground sediments mainly include *Hydrogenophaga*, *Thauera*, *Rhodobacter*, *Pseudomonas*, *Marinobacter*, etc. *Hydrogenophaga*, the dominant genus in both coal and RR samples, is a hydrogenophilic autotrophic bacterium capable of using

oxygen as a terminal electron acceptor for sugar metabolism [29]. Some species of this genus can degrade organic compounds such as polycyclic aromatic hydrocarbons [30], benzene, and meta- and paraxylene [31]. *Thauera* is a Gram-negative bacterium belonging to the *Beta-Proteobacteria*; most of the bacteria of this genus isolated so far can achieve denitrification and degradation of aromatic compounds [32] and have the ability to denitrify and secrete extracellular polymers [33]. *Rhodobacter* has a wide range of metabolic capabilities. One of the most studied species, *Rhodobacter sphaeroides*, can perform metabolisms such as photosynthesis [34], lithotrophy, and aerobic and anaerobic respiration [35]. *Pseudomonas* is a group of specialized aerobic Gram-negative bacteria, suitable for growth in moist environments; some species are known to be conditionally pathogenic. The most common species is *Pseudomonas aeruginosa* [36] and some species, such as *Pseudomonas putida,* have the ability to degrade organic substances such as toluene, xylene, and polycyclic aromatic hydrocarbons [37]. *Marinobacter* is usually isolated from marine environments [38,39] or oil-contaminated areas [40] and bacteria of this genus are salinophilic and have the ability to degrade petroleum hydrocarbons [41] and polycyclic aromatic hydrocarbons [42]. *Dietzia* can degrade alkanes [43], petroleum hydrocarbons [44], etc. *Thiobacillus*, a chemoautotrophic bacterium, is commonly found in mine drainage [45,46]. This type of bacteria can produce energy by oxidizing reduced sulfides and has the ability to oxidize ferrous iron. *Nitrospira* is a chemoautotrophic nitrite-oxidizing bacterium of great importance in the biogeochemical nitrogen cycle [47,48]. By analyzing the characteristics of the main genera, we found that most of the dominant genera in the underground sediment have the function of degrading organic matter, which may be attributed to the presence of a large amount of coal in the sediment. Organic matter in coal is mainly composed of complex ring-loaded hydrocarbons and aromatic compounds, etc. [49]. Therefore, such environmental conditions promote genera with organic matter metabolic functions, such as hydrocarbons and aromatic hydrocarbons, to become the dominant community.

4.2. Analysis of Differential Species and Their Influencing Factors in Different Group Sediment Samples

Significant differences in the microbial community structure exist between the coal and RR samples. Results of the Metagenomeseq analysis revealed that, out of the 1674 genera obtained from the underground sediment samples, 150 genera were significantly different in distribution between the two groups of samples, accounting for 18.01% of the total relative abundance of the library. The distribution of microorganisms was closely related to environment factors. Heat maps of the correlation between these significantly different major genera (average relative abundance >0.1% in at least one group sample) and environmental factors are shown in Figure 5. These distinct genera can be clustered into two groups, based on the clustering of correlations between genera and environment factors. The genera in cluster I mainly include *Hydrogenophaga*, *Rhodobacter*, *Thauera,* and *Methyloversatilis*, all of which have higher average relative abundances in the CR sediment than in the RR, probably because the environmental conditions in the coal roadway are more suitable for the survival of these microorganisms. Correlation heat maps show that the distribution of these genera in cluster I is negatively correlated with Cu and positively correlated with temperature, TP, and TS. As the heavy metal Cu inhibits cell metabolism and is toxic to the growth of microorganisms [49], it is commonly used as a fungicide, which may explain the negative correlation between the microorganisms in cluster I and Cu. In this study, the coal mines are mined at a depth range of −400 to −1000 m; the temperature of the underground environment is higher than that of the surface environment due to the influence of ground temperature [50]. During the coal mining process, heat is released, resulting in high temperatures in the coal mining roadway. The temperature of the coal roadway environment in this study ranges from 22 to 30 °C. Such temperature conditions are conducive to the growth and reproduction of microorganisms, which may explain the positive correlation between these genera and temperature in cluster I. Genera in cluster II include *Rubrobacter*, *Dietzia*, *Planctomicrobium*, *Pirellula,* and *Nesterenkonia*, which are

mainly found in rock roadway sediment. In contrast to the microorganisms in cluster I, the correlation heat map results show that the distribution of these genera in cluster II is positively correlated with heavy metals, possibly because these different genera in the rock roadway sediment have greater resistance to heavy metals and can adapt to relatively high concentrations of heavy metals. The rock group sediment contains higher levels of Mn, Fe, Cu, and As than the CR. In environments with high levels of heavy metals, microorganisms have gradually evolved adaptations to heavy metals [51].

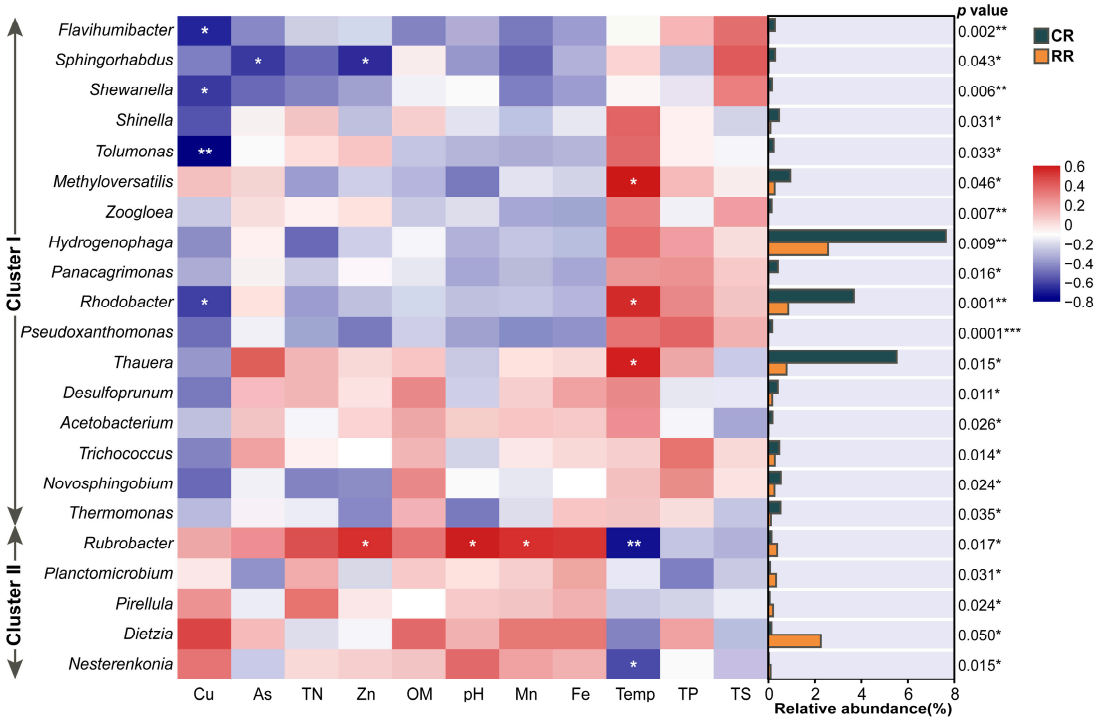

Figure 5. Heat map of the distribution of major genera with significant differences between groups and their correlation with environmental factors, with '*' indicating the significance of the correlation. Right columns show average relative abundance of major genera in CR and RR groups. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

4.3. Comparison of Microbial Community Functions

To investigate the functions of the microbial community in coal mine sediment, we used Tax4Fun, a metabolic function prediction method using SILVA as a reference database [26]. The prediction of the sediment obtained 6 level 1 functions and 41 level II functions. A total of 18 level II functions had relative abundances >1% (Figure 6a), which mainly included carbohydrate metabolism, amino acid metabolism, membrane transport, signal transduction, and energy metabolism, etc. Among them, the relative abundance of the top 11 functions together exceeded 80%. Statistical analysis showed that the CR samples were significantly different from the RR samples in the functions of membrane transport, metabolism of other amino acids, and folding, sorting, and degradation ($p < 0.05$). The enhanced membrane transport and other amino acid metabolic functions of microorganisms in the underground environment of the coal roadway may be an adaptation mechanism of microorganisms to the underground coal mine environment. In addition, the microorganisms of the RR samples were enhanced in translation, replication and repair, nucleotide metabolism, folding, sorting, and degradation compared with the CR samples. RR samples had a relatively high content of heavy metals, which were toxic to bacterial cells. Therefore, microorganisms in RR samples evolved effective repair functions by accelerating replication and reproduction to adapt to the external environment [52]. As we observed, the cell growth and death function were also highly expressed in RR sediment.

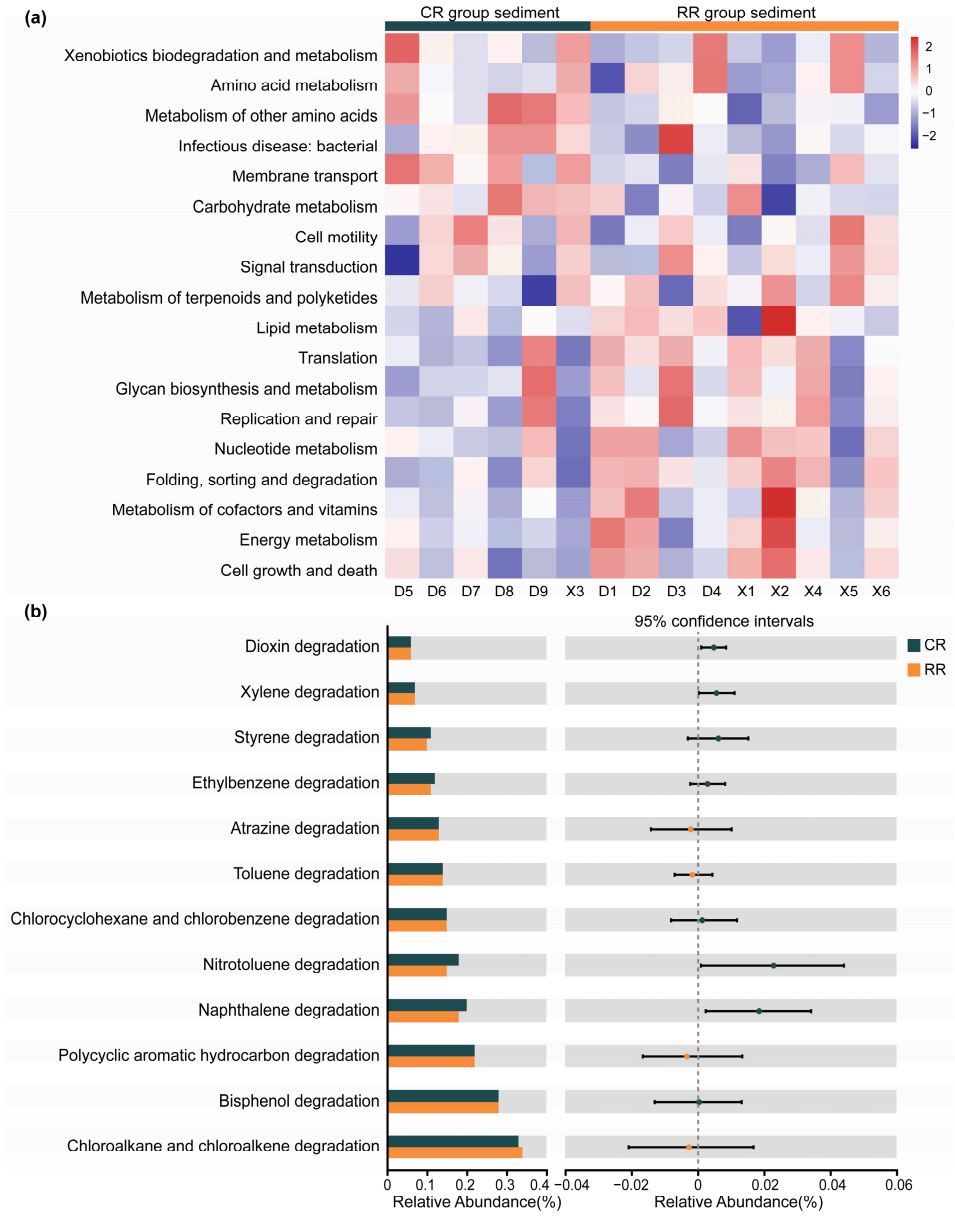

Figure 6. Heat map of the top 18 level II functions in underground coal mine sediment based on Tax4Fun functional prediction (**a**). Distribution and comparison of level III functions related to organic matter degradation in different group sediment samples (**b**).

From the preliminary analysis of microbial composition and properties, we found that a variety of microorganisms with organic matter degrading capabilities were present in the underground sediment. The main functions related to organic matter degradation were screened from the predicted level III functions and the relative abundances of these functions in different groups of samples were compared, as shown in Figure 6b. Statistical analysis showed that the relative abundances of microbial degradation functions of organic compounds such as dioxin, xylene, styrene, ethylbenzene, chlorocyclohexane and chlorobenzene, nitrotoluene, naphthalene, and bisphenol were higher in CR samples than in RR samples. RR samples showed the higher relative abundance of microbial degradation functions of atrazine, chloroalkane and chloroalkene, polycyclic aromatic hydrocarbon, and toluene than CR samples. During coal formation, the residues of various ancient plants undergo complex physicochemical changes, eventually forming a variety of complex organic substances present in the coal [3]. Along with coal mining, coal containing complex organic matter is gradually dispersed into various underground areas, such as roadways,

water tanks, drainage ditches, etc., which promotes the evolution and enrichment of microorganisms with organic matter degrading function in the underground environment. In addition, underground coal mining activities and mechanical equipment may discharge or leak substances such as lubricants, emulsions, and emulsified oils, etc. [53], which further stimulate the expression of organic matter degrading functions of microorganisms in the underground environment.

4.4. Prediction of Microbial Phenotypes in Underground Coal Mine Sediment

We used BugBase, a bioinformatics tool that can infer the phenotypes of the whole community based on the 16S rRNA gene [27], to gain insight into the functions of the bacterial community. A total of nine phenotypes were predicted for the microbial communities of the underground coal mine sediment samples, namely aerobic, anaerobic, contains mobile elements, facultatively anaerobic, forms biofilms, Gram-negative, Gram-positive, potentially pathogenic, and stress tolerant. The distribution of the relative abundance of each microbial phenotype in the coal and RR sediment is shown in Figure 7a. The prediction results showed that the percentages of aerobic and anaerobic microorganisms in the underground sediment were 51.01% and 18.42%, respectively. The dominance of aerobic microorganisms may be due to the continuous ventilation inside the producing coal mines, which is conducive to the growth of aerobic microorganisms. The microorganisms in the underground sediment were mainly Gram-negative bacteria, with an average percentage of 87.05%, which was much higher than that of Gram-positive bacteria (12.95%). According to the distribution of each microbial phenotype in the sediment samples, phenotypes such as facultatively anaerobic, potentially pathogenic, and stress tolerant were higher in the CR samples than in the RR samples, while other phenotypes were relatively higher in the RR samples (Figure 7a). Statistical analysis showed that of the nine predicted phenotypes, potentially pathogenic and stress tolerant showed significant differences in the distribution of the samples between groups ($p < 0.05$). The average percentages of potentially pathogenic and stress tolerant in the CR samples were 56.30% and 49.42%, respectively, while in the RR samples, they were 34.85% and 30.62%.

The potential pathogenicity of microorganisms in underground coal mine sediment is relatively high, suggesting that a variety of pathogens capable of causing disease may be present in the underground environment, indicating health risks to underground workers [54]. To identify microorganisms with potential pathogenic risk in sediment, we performed a statistical analysis of the contribution of potentially disease-causing microorganisms in coal and RR samples. The top ten contributing genera to potential pathogenicity in coal and RR sediment and their relative abundances are shown in Figure 7b,c. The main contributing genera in the CR samples included *Hydrogenophaga*, *Pseudomonas*, *Marinobacter*, *Methylophaga,* and *Geothermobacter*, with contribution percentages of 10.09%, 2.76%, 2.20%, 2.08%, and 1.90%, respectively. The most contributing genera in the RR group samples were *Hydrogenophaga*, *Thiobacillus*, *Geothermobacter*, *Sulfurifustis*, and *Luteimonas*, with the major contribution of 3.25%, 2.58%, 1.34%, 1.34%, and 1.17%, respectively. Pseudomonas has been reported as a typical conditionally pathogenic bacterium that can cause diseases such as traumatic infections, otitis media, and respiratory infections in humans [55]. In addition, we found that most of these genera with major contributions were Gram-negative bacteria. The outer membrane of Gram-negative bacteria consists of lipopolysaccharide (LPS) and other polysaccharides. Although such bacteria do not come into contact with humans under normal environmental conditions, cytokines in the human body react to the LPS surface of the bacteria, which in turn activates the body's innate immune response. The pathogenesis of this type of disease may be based on the inflammatory response of biological immune complexes to bacterial endotoxins, which can have toxic effects on the body [56].

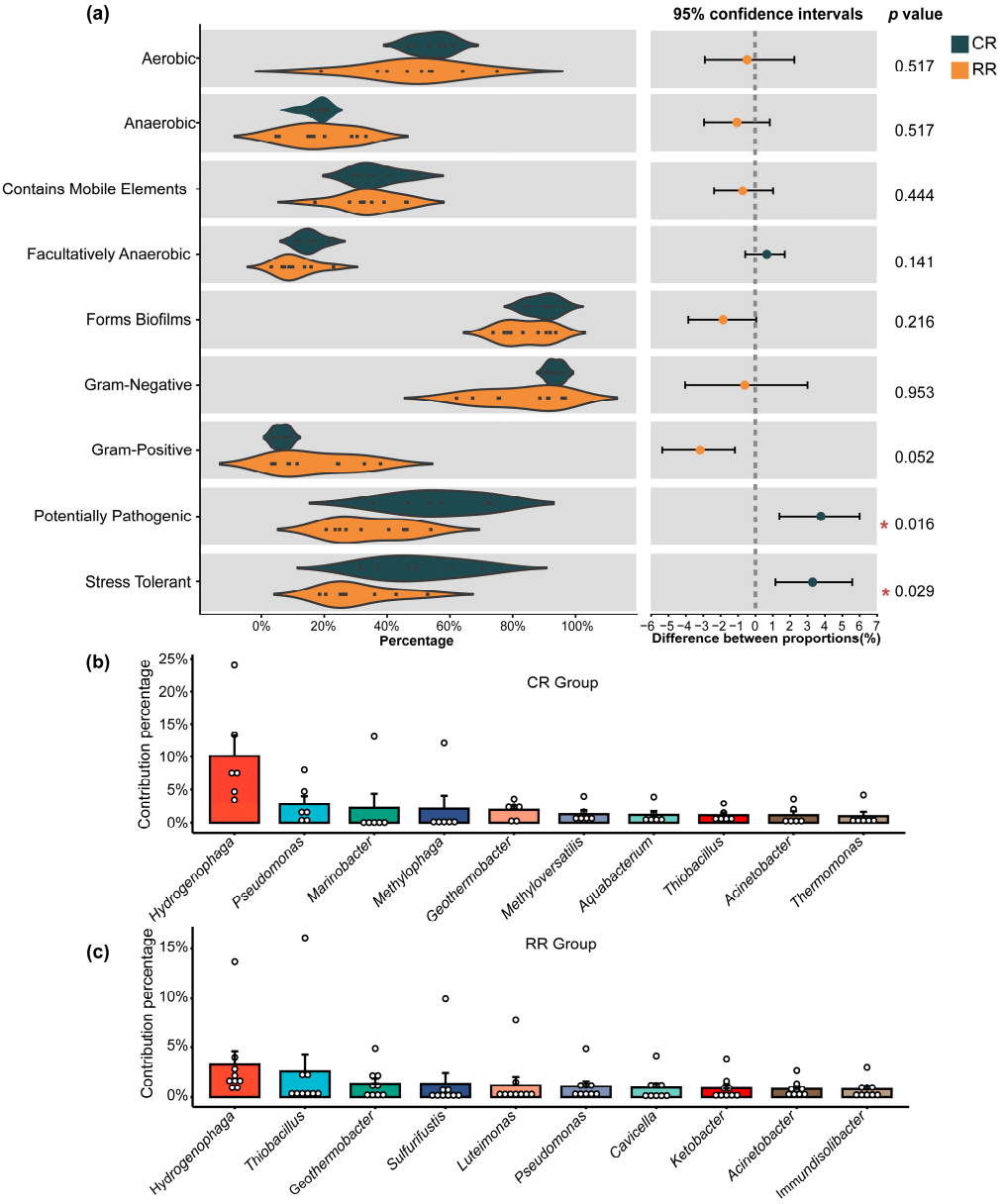

Figure 7. Distribution and comparison of the predicted microbial phenotypes in two groups of underground coal mine sediment (**a**) (* $p < 0.05$). Main contributing genera for potential pathogenic risk of microorganisms in sediment, CR (**b**), RR (**c**). Black points in (**a**) and the circles in (**b**,**c**) represent sampling points.

During the production of coal mining, dust and aerosols generated in underground workplaces can enter the human body through the respiratory system of the workers and thus pose a threat to the health of human beings. Currently, research into occupational health risks in underground mines focuses mainly on dust and other hazards, with less attention paid to the impact of environmental microorganisms. However, deep coal mines are susceptible to the growth of microorganisms, such as bacteria and fungi, due to the suitable temperatures and humidity, which, together with the introduction of microorganisms from coal production activities, result in a unique underground microbial community. Through the phenotypic prediction analysis of underground sediments, we have identified some potential pathogenic risks and disease-causing microorganisms in underground environmental samples. These microorganisms can attach to the surface of dust particles or form subsurface microbial aerosols, which can enter the human body and cause respiratory diseases such as asthma, allergies, and lower respiratory infections. Therefore, the potential

biological health risks posed by microorganisms in the downhole environment require more attention.

5. Conclusions

To determine the microbial community structure, metabolic functions, and phenotypic characteristics of long-term deep coal mine environments, we collected underground sediment from two producing mines in eastern China at mining depths of −400 to −1000 m. We found that the microbial diversity and community structure of the underground sediment from different coal mines were similar. A unique community of microorganisms has evolved in the underground coal mine environment, most of which can degrade organic pollutants such as polycyclic aromatic hydrocarbons (PAHs), benzene, toluene, and xylene, etc. The dominant genera of underground sediment included *Hydrogenophaga*, *Thauera*, *Pseudomonas*, *Rhodobacter, Dietzia,* etc. Results of the RDA analysis showed that the distribution of microorganisms in sediment samples was significantly influenced by TS (R^2 = 0.721, $p < 0.05$).

Significant differences were found in the microbial community structure of the RR and CR group sediment ($p < 0.05$). Genera that differed in the CR group sediment mainly included *Hydrogenophaga*, *Rhodobacter*, *Thauera, Methyloversatilis*, etc., and the distribution of these genera was negatively correlated with Cu and positively correlated with temperature, TP, and TS. Microorganisms in RR group sediment are highly resistant to heavy metals, and microorganisms accelerate replication and reproduction to adapt to the external environment by enhancing metabolic functions such as translation, replication and repair, nucleotide metabolism, folding, sorting, and degradation. Statistical analysis showed that the CR samples were significantly different from the RR samples in the functions of membrane transport and metabolism of other amino acids and folding, sorting, and degradation ($p < 0.05$). Microorganisms in the CR samples had high erect degradation functions for organic pollutants. Bugbase phenotypic predictions indicated high potential pathogenicity of microorganisms in underground coal mine sediment. The main contributing genera in the CR samples included *Hydrogenophaga*, *Pseudomonas*, *Marinobacter*, *Methylophaga,* and *Geothermobacter*, with contribution percentages of 10.09%, 2.76%, 2.20%, 2.08%, and 1.90%, respectively. For RR sediment, the main contributing genera were *Hydrogenophaga* (3.25%), *Thiobacillus* (2.58%), *Geothermobacter* (1.34%), *Sulfurifustis* (1.34%), and *Luteimonas* (1.17%), respectively. This study deepens the understanding of microbial communities in deep coal mine environments; however, the organic contamination and biological health risks of underground environments require extensive attention.

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