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Microbial structure, diversity, and function in saline soils of Belozem, Bulgaria: a metagenomic and enzymatic activity assessment

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ABSTRACT

Saline soils represent a major challenge to agricultural productivity, particularly in regions like Belozem, Bulgaria, where salinization is both naturally occurring and anthropogenically induced. This study investigates the microbial status of strongly saline soils through a combination of conventional microbiological methods and metagenomic sequencing. Soil respiration (SR), substrate induced respiration (SIR), colony-forming units (CFU), and enzymatic activities (β-glucosidase and dehydrogenase) were assessed alongside high-throughput 16S rRNA gene sequencing. Results indicate that increased soil salinity and gravimetric water content negatively affect microbial respiration and diversity. The microbial community was dominated by halotolerant taxa including Actinobacteriota, Proteobacteria, and Firmicutes. Soil respiration was significantly correlated with moisture, and SIR values revealed high microbial dormancy under saline stress. Enzyme assays indicated suppressed metabolic activity in high-salt environments, particularly in soils with the lowest pH and highest EC values. Metagenomic analysis revealed variations in alpha and beta diversity across the three soil types, reflecting salinity-induced shifts in microbial community structure and function. These findings highlight the ecological consequences of salinity on soil microbial dynamics and suggest that metagenomic approaches can offer valuable insights for managing saline-affected ecosystems.

Keywords: Soil bacteriome, Soil respiration, Enzymes, Halotolerant bacteria, 16S rRNA sequencing

INTRODUCTION

Soil salinization is a major environmental and agricultural challenge globally, affecting approximately 20% of irrigated land and posing a substantial threat to food security, ecosystem function, and sustainable land use practices. Globally, more than 930 million hectares of land are affected by salinity, with about 30.7 million hectares located in Europe alone, where both natural and anthropogenic processes contribute to this degradation (FAO, 2021; Rengasamy, 2006). In Bulgaria, soil salinity impacts around 55,000 hectares, particularly in low-lying areas such as Belozem, where historical rice cultivation and inadequate drainage infrastructure have exacerbated the problem (Andreeva and Poushkarov, 2020; Penov et al., 2011).

Saline soils are characterized by high concentrations of soluble salts primarily sodium chloride and sulphates with an electrical conductivity (EC) of ≥4 dS/m and a pH typically between 7.0 and 8.5 (IUSS Working Group WRB, 2022; Richards, 1954). These conditions severely restrict plant growth by creating osmotic stress, nutrient imbalances, and ion toxicity, leading to physiological and metabolic dysfunctions (Machado and Serralheiro, 2017; Munns and Tester, 2008). Moreover, salinity alters the physical structure of soils by dispersing clay particles, reducing porosity and permeability, and impairing water infiltration (Setia et al., 2013; Wong et al., 2008).

In addition to its impact on plants and physical properties, salinity exerts a profound influence on soil microbial communities known to be key drivers of soil biogeochemical processes. Microorganisms mediate essential functions such as organic matter decomposition, nutrient cycling, and the regulation of soil enzyme activities (Sahu et al., 2017; Verma et al., 2017). These microbial driven processes are critical for maintaining soil fertility and overall ecosystem resilience, especially under stress conditions. High salt concentrations disrupt

72 microbial community composition by selecting for halotolerant or halophilic species and
73 reducing overall diversity (Lozupone and Knight, 2007; Rath and Rousk, 2015). Several studies
74 have reported reductions in microbial biomass, activity, and functional potential in saline soils
75 (Egamberdieva et al., 2010; Yan and Marschner, 2013).

76 Soil enzymes, such as dehydrogenase and β-glucosidase, serve as sensitive indicators of
77 microbial metabolic activity and soil health (Dotaniya et al., 2019). Their activities are often
78 suppressed under saline conditions due to osmotic stress and reduced microbial biomass (Rietz
79 and Haynes, 2003; Singh, 2016). Dehydrogenases, involved in oxidative reactions during
80 microbial respiration, and β-glucosidase, responsible for cellulose degradation, both provide
81 insights into how microbial functioning is impacted by salinity-induced stress (Salazar et al.,
82 2011; Zhao et al., 2010). Furthermore, soil respiration (both basal and substrate-induced)
83 provides a useful proxy for measuring microbial metabolic response under varying soil
84 conditions, including moisture and salinity (Anderson, 1982; Setia et al., 2013).

85 Advances in high-throughput sequencing, particularly 16S rRNA gene-based
86 metagenomics, now allow detailed exploration of microbial communities in challenging
87 environments such as saline soils. These techniques offer insights into microbial taxonomic
88 composition, phylogenetic relationships, and potential ecological functions (Caporaso et al.,
89 2010; Zhang et al., 2019). Despite the recognized role of salinity in shaping microbial
90 communities globally, there is a paucity of data concerning its effects in Eastern Europe, and
91 particularly in Bulgaria, where soil salinization is increasingly problematic due to climate change
92 and suboptimal land management practices (Daliakopoulos et al., 2016; Montanarella, 2007).

93 This study focuses on the microbial characterization of saline soils in Belozem, Bulgaria,
94 an area historically affected by both primary and secondary salinization. By integrating

conventional microbiological assessments (soil respiration, CFU counts, and enzyme activities) with metagenomic sequencing, we aim to: (i) evaluate how soil salinity influences microbial abundance, diversity, and function; (ii) explore phylogenetic relationships within microbial communities; and (iii) assess the relationship between microbial activity and soil physicochemical parameters such as moisture and EC. The outcomes of this research will contribute to a better understanding of saline soil microbiomes and inform sustainable land management practices in salt-affected agroecosystems.

MATERIALS AND METHODS

Study Area and Soil Sampling

The study was conducted in Belozem (42°11′47″N, 25°2′44″E), located in the Upper Thracian Lowland near Plovdiv, Bulgaria. This area is known for its pronounced salinization, with approximately 40% of the land exhibiting strong salt accumulation, primarily as a result of poor drainage, high groundwater tables, historical irrigation practices, and natural geomorphological features (Andreeva and Poushkarov, 2020; Penov et al., 2011). The soils are predominantly Solonetz, Luvisols, and Vertisols, characterised by montmorillonite-rich clay and poor permeability, making them highly susceptible to secondary salinization (IUSS Working Group WRB, 2022). All soil samples collected from Belozem were classified as strongly saline soil based on the Richards (1954).

Three agricultural fields with different land-use histories (irrigated rice, mixed crops with liming, and abandoned farmland) were selected. During the May 2024, four different rhizosphere soil samples were collected from each site. Each of these samples was composed by three subsamples of weed plants. The samples were taken from the upper layer between 5 and 15 cm

depth using a sterile stainless-steel auger, homogenizing the soil of each sample after sampling. To preserve microbial integrity, samples were placed in sterile polyethylene bags, kept on ice, and transported immediately to the laboratory for analysis (Doran and Parkin, 1994).

Soil Physicochemical Properties

Soil pH and electrical conductivity (EC) were measured using a 1:5 soil-to-water extract (w/v). Samples were shaken on an orbital shaker for 1 hour, allowed to settle for 2 hours, and then analyzed with a calibrated pH/EC meter (Mettler Toledo FiveEasy™) (Rhoades, 2018). Gravimetric water content (GWC) was determined by oven-drying 10 g of fresh soil at 105°C for 24 hours and calculating the moisture percentage as per standard protocols (Gardner, 2018).

Soil Respiration and Soil-Induced Respiration

Basal soil respiration (SR) and soil-induced respiration (SIR) were determined according to Anderson (1982). For each sample, 50 g of moistened soil on air-dried basis were used for the experiment after sieving (2mm mesh). The moistening was made first measuring the water content to a small portion of soil with a drier and adjusting the water content to the original soil till 60% WHC, gravimetrically. Further the soil was incubated in a sealed glass jar with a beaker containing 20 mL of 0.05 M KOH to absorb CO_2. After 6 hours of incubation at 25°C, the remaining KOH was precipitated with $BaCl_2$ and titrated with 0.1 M HCl using phenolphthalein as an indicator. To estimate the potential activity of dormant microbial biomass, an identical setup was amended with 1% glucose (SIR). Respiration rates were expressed as mg CO_2-C released per g soil per hour. This dual approach distinguishes metabolically active from dormant microbial populations and is widely accepted as a proxy for microbial health under stress conditions such as salinity (Zibilske, 2018).

Microbial Enumeration by CFU Counting

Soil microbial populations were quantified using the serial dilution and spread plate technique on five selective media: Tryptic Soy Agar (for total heterotrophs), Jensen's Agar (nitrogen-fixers), Actinomycete Isolation Agar, Yeast Extract Agar (for fungi and general heterotrophs), and Pikovskaya's Agar (phosphate solubilisers). Serial dilutions were prepared up to 10^{-5}, and 0.1 mL aliquots were spread onto agar plates in triplicates. Plates were incubated at 28°C for 48–72 hours, and colony-forming units (CFUs) were expressed as $\log_{10}$ CFU per gram of dry soil. Media compositions followed protocols described in (MacFaddin, 2000).

Enzymatic Activity Assays

β-Glucosidase Activity

β-glucosidase activity was measured using the method of Eivazi and Tabatabai (1988), based on the release of p-nitrophenol (pNP) from p-nitrophenyl-β-D-glucoside. 1 g of soil was incubated with 4 mL modified universal buffer (pH 6.0), 1 mL of 25 mM substrate, and 0.25 mL toluene. After incubation at 37°C for 1 hour, 1 mL of 0.5 M $CaCl_2$ and 4 mL of 0.1 M THAM buffer (pH 12) were added to terminate the reaction. The filtrate was analyzed spectrophotometrically at 400 nm. Enzyme activity was expressed as µg pNP released per g soil per hour.

Dehydrogenase Activity

Dehydrogenase activity was determined using 2,3,5-triphenyltetrazolium chloride (TTC) as the electron acceptor, following (Thalmann, 1968). 5 g of fresh soil was incubated with 5 mL TTC-TRIS buffer at 30°C in the dark for 24 hours. The produced triphenyl formazan (TPF) was extracted with acetone and quantified at 485 nm using a spectrophotometer. Results were

161 expressed as µg TPF per g soil per 24 h. This assay is widely recognized as an index of total
162 microbial oxidative activity (Moeskops et al., 2010).
163
164 **DNA Extraction and Metagenomic Sequencing**
165 DNA was extracted from 0.5 g of each soil sample using the DNeasy PowerSoil Kit
166 (Qiagen), following the manufacturer's protocol. DNA quality and quantity were assessed by
167 agarose gel electrophoresis and NanoDrop spectrophotometry (Thermo Scientific). The V4
168 hypervariable region of the 16S rRNA gene was amplified using primers 515F/806R Caporaso et
169 al. (2011), and amplicons were sequenced on an Illumina MiSeq platform (2×250 bp paired-end
170 reads) at Novogene (UK). Raw reads were demultiplexed, quality filtered, and merged using
171 QIIME 1.9.1 (Caporaso et al., 2010). Operational taxonomic units (OTUs) were clustered at 97%
172 similarity using UCLUST (Edgar, 2013). Taxonomic assignment was performed against the
173 SILVA database. Chimeric sequences were removed with USEARCH, and alpha diversity indices
174 (Shannon, Chao1, ACE) were calculated using Mothur. Chao1 represents the species richness
175 (i.e., the number of taxa presented), focusing on rare species, while the Shannon index combines
176 two key parameters: richness (how many species are present) and evenness (how evenly
177 distributed they are and whether the species are present in similar proportions or dominated by a
178 few). In addition, ACE index focuses on estimating species richness, with attention to the rare
179 taxa.The raw sequence data have been deposited in the NCBI Sequence Read Archive (SRA)
180 under BioProject accession number PRJNA1120469 (Belozem saline soils microbiome). The
181 SRA submission ID is SUB14508197, and the dataset includes three biosamples collected on
182 June 5, 2024. Processed data are publicly accessible through the SRA portal.
183

184 **Diversity and Statistical Analysis**

185 Alpha diversity (Observed species, Shannon, Chao1, ACE – Abundance-based Coverage
186 Estimator) and beta diversity (Bray-Curtis, UniFrac) metrics were calculated to assess species
187 richness and community composition among samples. Principal Component Analysis (PCA) and
188 Unweighted Pair Group Method with Arithmetic Mean (UPGMA) clustering were used to
189 visualize differences in microbial communities across sites (Lozupone et al., 2011). Statistical
190 analysis was conducted using R (version 3.6.1) and SPSS (version 25). One way ANOVA was
191 used to compare the physical properties of the soils, and Pearson's correlation was applied to
192 examine relationships between respiration and moisture ($p < 0.05$ was considered significant).
193 Differences in enzyme activity and microbial counts were revealed using the standard error
194 calculation.

195

196 **RESULTS**

197 **Soil Physicochemical Properties**

198 The values of electrical conductivity (EC) ranging from 9.54 to 13.56 dS m^{-1} (Table 1).
199 The pH varied significantly between sites, from moderately acidic (6.12) to slightly alkaline
200 (7.23), and gravimetric water content (GWC) ranged from 23.99% to 58.81%. The irrigated rice
201 field (Belozem.1) showed the highest EC and GWC, indicating excessive moisture retention in a
202 poorly drained environment, while the limed field (Belozem.2) had moderately high EC and
203 significantly lower GWC. These variations suggest site-specific microenvironments that may
204 influence microbial community structure and function.

205

206 **Soil Respiration and Soil-Induced Respiration**

207 The emission of carbon dioxide (CO_2) known as soil respiration was used to assess the
208 impact of salinity on microbial activities within the soil. Induction with glucose (SIR) was done
209 to identify the number of dormant microorganisms in the soil. Soil respiration was significantly
210 different in all the soil samples used for this study and was generally low compared to soil
211 induced respiration (SIR) (Figure 1). The CO_2 emitted (SR) by the soils of Belozem.2 was higher
212 compared to the other samples, however, after induction with glucose, SIR tripled, indicating a
213 significant level of microbial dormancy, nevertheless, the magnitude of increase (SIR) was low
214 compared to that of Belozem.2 and 3 (Figure 1a). The lowest CO_2 was emitted by soils of
215 Belozem.1 and 3 (SR) however, an immediate increase was observed when these soils were
216 induced with glucose (SIR). More dormant microorganisms were present in these soils indicating
217 the increment in SIR.

218 Statistically, the correlation between gravimetric water content and soil respiration was
219 significant ($p<0.008$). Gravimetric moisture content negatively correlated with soil respiration
220 indicating that as soil moisture increases soil respiration decreases and vice versa (Figure 1b).

221

222 **Microbial Abundance and Functional Group Composition**

223 The soil microbial count did not differ significantly among the samples; however, a
224 substantial number of microorganisms were present in these saline soils. Belozem.1, with the
225 highest salt content, recorded the lowest counts of nitrogen-fixing bacteria and actinomycetes but
226 had the highest levels of P-solubilizing bacteria (Figure 2). In Belozem.2, P-solubilizing bacteria
227 were more abundant than any other soil microorganisms, and actinomycetes were also more
228 prevalent compared to all other soil samples. Belozem.3, with the highest pH value and lowest

229 salt content, exhibited substantially higher levels of halophilic bacteria than all other samples.
230 The presence of P-solubilizing bacteria slightly suppressed the growth of Halophilic bacteria in
231 both Belozem 1 and 2 whereas the presence of the high levels of Halophilic bacteria suppressed
232 the growth of P-solubilizing bacteria in Belozem. 3.
233
234 **Soil Enzymatic Activity**
235 The activity of dehydrogenase in the saline soils of Belozem was highly significant
236 ($p=0.0004$), while β-glucosidase activity was not significantly different. The Belozem.3 site,
237 which had the lowest salt content and EC of 9.43 dS m^{-1}, exhibited the highest dehydrogenase
238 (Figure 3a) and β-glucosidase (Figure 3b) activities compared to all other sample sites. However,
239 Belozem.2. site which had an intermediate salt content, EC and pH recorded the lowest
240 dehydrogenase and β-glucosidase activities.
241
242 **Microbial diversity, richness, and structure in saline soils of Belozem**
243 The analysis of alpha diversity revealed pronounced differences in the composition and
244 structure of soil microbial communities among the studied samples (Figure 4 a and b). The
245 Shannon index, which integrates both species richness and evenness, also exhibited an increasing
246 trend across the samples. The lowest value was observed in Belozem.1 (6.07), whereas higher
247 values were recorded in Belozem.2 (7.35) and Belozem.3 (7.87). These results imply that, in
248 addition to reduced richness, the microbial community in Belozem.1 is likely characterized by
249 lower evenness, potentially reflecting the dominance of a limited number of taxa. In contrast, the
250 elevated Shannon index values in Belozem.2 and Belozem.3 indicate more evenly distributed
251 microbial populations, with a more balanced representation of taxa. Consistent with these

findings, the Chao1 index, applied as a robust estimator of species richness, demonstrated substantially higher values in Belozem.3 (1514.66) and Belozem.2 (1457.76) compared to Belozem.1 (902.49). This indicates a markedly greater number of observed and predicted taxa in the former two samples, suggesting a more complex and taxonomically rich microbial assemblage. The combined interpretation of both diversity indices highlights a clear gradient in microbial diversity and community organization, following the order Belozem.1 < Belozem.2 < Belozem.3. Notably, Belozem.3 exhibits the highest levels of both richness and evenness, suggesting a highly diverse and structurally complex microbial ecosystem. Belozem.2 displays intermediate characteristics, while Belozem.1 is distinguished by comparatively reduced diversity and a simpler community structure. These observed differences in alpha diversity may reflect underlying variations in environmental conditions, resource availability, or soil physicochemical properties, which can influence microbial colonization, survival, and community dynamics

The comparison between soil microbial communities within the sampling sites was done using the Principal Component Analysis (PCA). Two principal component factors (PCF) in relation to the OTU were used to explain total variation of 53.84% and 46.16% (Figure 4c). The analysis revealed that the three sampling sites were distinctly far from each other, indicating a vast difference in specie composition, nevertheless, Belozem.2 and 3 had a higher similarity compared to Belozem.1. However, Belozem.1 and 3 were more decentralized than Belozem.2.

The Beta diversity analysis of soil microbial community composition based on the Weighted Unifrac distance matrix indicated that Belozem.2 and 3 were more similar in terms of microbial structure with an index of 0.548 (Figure 4d). However, microbial community was more dissimilar in saline soils of Belozem.1 and 3 with a β-diversity index of 0.656.

275

276 **Taxonomic classification of microbes in saline soils of Belozem**

277 The composition of microbial community analysis indicated a significant difference
278 among the three salt affected soils. At the phylum level, 10 out of 135 phyla were common to all
279 samples. Additionally, the microbial composition of these ten dominant phyla differed
280 significantly ($P < 0.05$) between Belozem.1 to Belozem.3 (Figure 5a). Generally, Firmicutes
281 (26%), Proteobacteria (22.5%) and Actinobacteriota (21.5%) were the most prevalent Phyla in all
282 sampling sites. The other seven predominant phyla in the three sampling sites included
283 Gemmatimonadota (6.9%), Acidobacteriota (7.3%), Verrucomicrobiota (4.4%), Bacteroidota
284 (2.9%), Chloroflexi (3.2%), Planctomycetota (1.3%), Crenarchaeota (0.6%), and others (3.4%).
285 A very interesting trend occurred at this level among the individual sampling sites as an increase
286 in Firmicutes (33.3%), a decrease in Proteobacteria (24.4%) in Belozem.1, a decline in
287 Firmicutes (9.3%), and increase in Proteobacteria (29%) in Belozem.3. Another notable
288 observation was the equal amounts of Proteobacteria and Actinobacteriota (14%) in salt affected
289 soils of Belozem.2.

290 At the class level, the average relative abundance of Bacilli (26%) was very high,
291 nevertheless, the individual sites differed significantly (Figure 5b). In Belozem.3,
292 Alphaproteobacteria (19.5%) was the most dominant followed by Thermoleophilia (15.7%)
293 whereas Belozem.1 and 2 had similar dominance levels of Bacilli (33-35%). However,
294 Alphaproteobacteria (11.9%) was more predominant in Belozem.1 than Belozem.2 (6.6%). On
295 average, the increment in Bacilli (26%) resulted in the decline of Alphaproteobacteria (12.7%) at
296 the class level.

297 A higher abundance of Bacillales (25.8%) was found at the order level nevertheless a
298 sizable amount microbes classified as “others” (35.1%) was significantly the highest (Figure 5c).
299 Additionally, there was a notable relationship between Sphingomonadales and Gaiellales, as both
300 were present at a very similar rate 6% in these salt affected soils.

301 At the family level, microbial communities of *Bacillaceae* (22.6%) was very high,
302 although it was somewhat lower compared to its abundance at the phylum (26%), class (26%),
303 and order (25.8%) level (Figure 5d). Additionally, the family *Gemmatimonadaceae* (5.3%),
304 *Sphingomonadaceae* (6.4%), and *Planococcaceae* (3.2%) were also abundant in all the salt
305 affected sites. A sizable amount of the 16S rRNA gene sequences of microbial populations were
306 categorized as "others" (50.2%) since they belonged to several families at smaller quantities.
307 However, the abundance of others were higher in these saline soils at the family and order level
308

309 **Evolutionary relationship of microorganisms in saline soils of Belozem**

310 Aligned representative sequences were used to generate a phylogenetic tree, serving as an
311 evolutionary map to observe microbial evolution up to the genus level. A total of 103 genera
312 from the 10 dominant phyla were selected from the salt affected soils of Belozem to further
313 investigate the phylogenetic relationships within the genus (Figure 6). The majority of the
314 microorganisms in these saline soils at the genera level belonged to the phyla Proteobacteria and
315 Actinobacteriota. *Bacillus*, which evolved from the phylum Firmicutes was the predominant
316 genus in Belozem.1 whereas *Sphingomonas* from the phylum Proteobacteria was dominant in
317 Blozem.3 (6a). In Belozem.2, *RB41* and *Halobacillus* from the phyla Acidobacteriota and
318 Firmicutes respectively were more abundant. Furthermore, since each experimental site had its
319 own land uses and microclimate there were certain unique microbes identified to be site-specific.

320 *Subgroup_10, Pseudomonas, Psychrobacillus, Phyllobacterium, Dongia,*
321 *Candidatus_Xiphinematobacter* and *Rhodcoccus* were among the bacteria that were unique to
322 Belozem 1. For Belozem.2 these site-specific microbes included *Halobacillus, Azotobacter,*
323 *Candidatus_Nitrosophaera* and *Stenotrophomonas*. Lastly Belozem.3 included
324 *Paenisporosarcina, Angustibacter,* and *Porphyrobacter*. Some unique genera such as
325 *Subgroup_10, Haliangium, Nitrospira* and *Tumibacillus* evolved directly without following the
326 typical taxonomic pathways. The members of Firmicutes in saline soils of Belozem include
327 Bacilli, Bacillales, Bacillaceae, and *Bacillus* (Figure 6b) in conformity with NCBI database.
328
329 **Taxonomic Abundance Cluster Heatmap at the specie level**
330 A heatmap was created based on the abundance of the top 35 species in all samples to
331 determine similarity and differences among sampling sites (Figure 7). At the specie level the
332 dominant phyla were Proteobacteria and Actinobacteriota. *Rhizobium phaseoli, Pseudomonas*
333 *boreopolis, Arthrobacter crystallopoiestis, Psychrobacillus psychrodurans, Bacillus anthracis,*
334 *Paenibacillus sp JDR-2, Rhodococcus wratislaviensis, among others* were unique to Belozem.1
335 whereas *Bacillus decolorationis, Stenotrophomonas maltophilia, Sphingobacterium multivorum,*
336 *Aquabacterium citratiphilum, Mitsuaria chitosanitabida, Chryseobacterium sp IHB B 17019,*
337 *Sorangium cellulosum, Gemmatimonadetes bacterium LX87, among others were* unique to
338 Belozem.2. *Bacterium Ellin (6543, 5290, and 6517), Flavisolibacter ginsengisoli, and*
339 *Gemmatimonadetes bacterium WY71* were distinct to Belozem.3.
340
341

DISCUSSION

The soils investigated in this study represent typical examples of secondary salinization, largely driven by anthropogenic factors such as irrigation with poor-quality water and insufficient drainage, as previously reported for Belozem (Manolov et al., 2008). Our results confirm that high electrical conductivity (EC) and soil moisture content significantly impair microbial activity, particularly as reflected in soil respiration (SR) metrics. SR was consistently lower than substrate-induced respiration (SIR), which indicates a high proportion of dormant microbial biomass. This corroborated with the findings of Petkova et al. (2020) who reported that most microorganisms at the SR stage are dormant and therefore induction with glucose at the SIR stage stimulates their release of CO_2. Research conducted by Li et al. (2018) linked low SR to reduced microbial activities, suggesting that soil salinization might negatively impact microbial processes. Similarly, studies conducted by Chowdhury et al. (2011); Setia et al. (2011a,b) reported significantly low SR in saline soils, attributing this to the harmful effects of salt on soil microorganisms. Therefore, it is possible that the high level of salinity in soils of Belozem could have inhibited microbial release of CO_2 resulting in extremely low SR. Among the individual samples SR was significantly higher in Belozem.2, followed by Belozem.3, and Belozem.1 (Figure 1a). This is likely due to the low Gravimetric Water Content (GWC) in Belozem.2 (Table 1), as high soil moisture negatively affects respiration. This relationship was further confirmed through correlation analysis, which showed a negative correlation between soil moisture and respiration (Figure 1b). This finding aligns with Meena et al. (2020), who also reported a negative correlation between soil moisture and temperature with soil respiration. Additionally, the level of salinity in Belozem.1 was higher than all the other samples; however

we observed a very low SR indicating that high salt content in the soil can reduce CO_2 release by the soil. Similar findings were reported by (Mavi et al., 2012; Wong et al., 2008).

According to the results from the Colony Forming Units (CFU), Phosphate-solubilizing bacteria emerged as the most dominant followed by Halophilic bacteria (Figure 2). P-solubilizing bacteria has been reported by several researchers to thrive more in saline environments than other microorganisms (Herlemann et al., 2011; Hu et al., 2023; Iftikhar et al., 2024). Several possible explanations exist for this phenomenon, as various bacterial communities are known to occupy several ecological niches like saline soils Herlemann et al. (2011), and they tend to solubilize phosphorus under challenging conditions (Iftikhar et al., 2024). Furthermore, given that the saline soils used in this study were from agriculturally active fields, it is possible that the application of phosphate fertilizers, as suggested by Yu et al. (2011), may have promoted the growth and development of phosphate-solubilizing bacteria in the soil. Sabet et al. (2009) speculated that halophilic bacteria thrive in low saline soils. However, this study contradicted their findings, as it discovered a greater abundance of halophilic bacteria in soils with higher levels of salinity. Similar findings were made by (Delgado-García et al., 2018).

The enzymatic activity results indicated that there were more dehydrogenase activities and β-Glucosidase in the soils of Belozem.3 compared to Belozem.1 and Belozem.2 (Figure 3). However, the influence of soil salinity on β-Glucosidase was not significant in this study and similar findings were reported by (Sritongon et al., 2022). In contrast, dehydrogenase activity was highly significant, particularly in Belozem.3, which recorded the highest levels and Belozem.2 which recorded the lowest. In our observations from these two experimental fields, we noted that areas with higher soil moisture content (GWC from Table 1) tended to exhibit higher dehydrogenase activity (Belozem.3) and areas with lower GWC exhibited lower

dehydrogenase activity (Belozem.2). This aligns with the research findings of (Tomar and Baishya, 2020) who reported that dehydrogenase tends to thrive in areas with higher moisture content. However, there are different opinions about the effect of soil moisture on dehydrogenase activity. Xie et al. (2017) suggested that dehydrogenase activity increases with soil moisture, while Wolinska and Stepniewska (2012) found the opposite, stating that increased moisture leads to decreased dehydrogenase activity. Therefore, there is a possibility that the highest and lowest dehydrogenase activity observed in Belozem.3 and Belozem.2 may be associated with its soil water content. This is because microbes utilize moisture for various metabolic reactions, and dehydrogenase enzymes are integral to these processes as they facilitate the transfer of hydrogen from organic substrates to inorganic acceptors (Zhao et al., 2010).

Microbial diversity and structure are known to fluctuate based on factors such as soil pH, temperature, organic content, and water availability (Wood et al., 2017). Studies using 16S rRNA sequencing have shown that microbial communities are particularly sensitive to different land use practices (Daquiado et al., 2016). In this study we demonstrated how microorganisms differ across the saline soils with similar levels of salt. Soil salinity affects structure, diversity and richness of microorganisms therefore; alpha diversity and beta diversity analysis were conducted in all the sites to determine microbial diversity, abundance and distribution. From the results Belozem.3 and Belozem.2 had a very high microbial community structure with a Shannon diversity index of 7.87 and 7.35 respectively (Figure 4a). This is an indication that microbial species are more evenly distributed creating a well-balanced microbial ecosystem in these saline conditions. This finding from the two sites is an indication that saline soils with similar levels of salt have a similar ecosystem distribution of microorganisms. This corroborated the findings of Yang et al. (2016) who reported that salinity significantly shapes microbial community structure.

410 According to the Chao1 index, Belozem.1 had the lowest evenness of the microbial composition
411 which is an estimate based on the total number of taxa present in a community (Figure 4b). This
412 indicates that salt affected soils of Belozem.1 contain a lower variety of microbial species.
413 Therefore, saline soil of Belozem.1 has a lower microbial diversity that could be important for
414 various ecological, including ecosystem functions. The results obtained contrasted with the
415 findings of Mukhtar et al. (2018) who identified a more diverse microbial communities in the
416 rhizosphere of moderate to high soils. The principal component analysis (PCA), a component of
417 beta diversity, indicated that microbial composition based on OTU across Belozem.3 and
418 Belozem.2 were very similar whereas Belozem.1 was highly dissimilar compared to the other 2
419 sites (Figure 4c and d). The similarity of Belozem.3 and Belozem.2 align with the general
420 consensus that microbial diversity tends to be low and similar across various extreme
421 environments (Smith et al., 2006). However, in the case of Belozem.1, they differed from the
422 observations made by Hollister et al. (2010), who identified a more diverse and dissimilarity of
423 microbial taxa in some saline soils.

424 Microbial communities that dominated saline soils of Belozem at the phylum level were
425 Firmicutes (26%), Proteobacteria (22.5%) and Actinobacteriota (21.5%) (Figure 5a). This
426 corroborated the findings of (Bhatt et al., 2018) that also identified Firmicutes as the most
427 dominant phyla and its correspondent Bacillus as its genera under saline conditions. These
428 findings were also within the discovered range of the phyla present in saline soils around the
429 globe as meta-analysis by Ma and Gong (2013) revealed that the majority (90%) of the bacterial
430 sequences in saline soils belonged to six dominant phyla, namely Proteobacteria,
431 Actinobacteriota, Firmicutes, Acidobacteria, Bacteroidetes, and Chloroflexi. In saline soils of
432 Belozem.1 and 2, Firmicutes found to have a Gram-positive cell wall structure emerged as the

predominant phyla; however, its microbial communities were not uniformly distributed across the different taxonomic levels. Previous literature suggested that this phylum is generally high in low saline environments (Ramette, 2007). However, Belozem.1 and 2 which had an EC of 13.54 dS m^{-1} and 11.32 dS m^{-1} respectively (Table 1) indicating high salinity levels yet it supported the growth and abundance of the Firmicutes. Therefore, these findings did not corroborate with that of (Ramette, 2007) nevertheless, similar findings were di scovered by (Fan et al., 2023). It was also reported by Kumar et al. (2011) that different communities of the phylum Firmicutes occupy various agricultural niches, enhancing crop productivity by producing phytohormones, antibiotics, solubilizing and mobilizing phosphate, fixing atmospheric nitrogen (N_2), and releasing ammonia (NH_3). Nevertheless, these strains can survive under challenging environmental conditions (abiotic stress such as salinity, drought, heat, cold, metal-rich, or acidic soils) for extended periods, awaiting favorable conditions to thrive (Banik et al., 2018). Proteobacteria, found to be one of the most prevalent bacterial taxa in saline soils was identified as the second most prevalent phylum followed by Actinobacteriota in saline soils of Belozem. These two phyla had the most dominant representatives at the genus and specie level indicating a good adaptation mechanisms built by their communities throughout their evolution process (Figure 5). The high prevalence of Proteobacteria and Actinobacteriota in saline soils of Belozem is linked to their capacity to flourish in highly saline conditions. These bacteria have been reported to produce various extracellular hydrolases, which break down and transform external organic matter into soluble forms of phosphorus, nitrogen, potassium, and other elements (Liu et al., 2024). This process is crucial for the mineralization of organic matter. Various studies have demonstrated the presence of certain bacterial phyla which were previously not associated with saline conditions but are currently thriving in saline soils. These include Nitrospira,

Deferribacteres, Cyanobacteria/Chloroplast, Gemmatimonadota, Planctomycetes, BRC1, Verrucomicrobiota, Tenericutes, Spirochaetes, WS3, and Chlorobi (Liszka et al., 2012). Interestingly, this research identified Gemmatimonadota (6.9%), and Verrucomicrobiota (4.4%) to be thriving in saline soils of Belozem. It is possible that these microbes have built a high tolerance to salt and are gradually living and evolving in the salty conditions. Relative abundance of 'others' at the order and family level were higher than all the identified microbial communities (Figure 5c and d). This is an indication that microbial communities in saline soils of Belozem are much more complex at the order and family level. The genus *Bacillus* was prevalent in the saline soils of Belozem.1, possibly due to its spore-forming capabilities and Gram-positive cell walls (Figure 6) (Schimel et al., 2007). Bacillus species play a crucial ecological role in biogeochemical cycles across various ecosystems, including marine waters and saline soils. They enhance plant growth, produce valuable industrial enzymes such as proteases, amylases, cellulases, and lipases, and participate in the bioremediation of various toxic chemicals and pollutants in saline environments (Mukhtar et al., 2018). Furthermore, *Bacillus* is significant in studying enzymes that tolerate high salt levels and metabolic processes that aid in cleaning pollutants from salty soils (Liszka et al., 2012). Strains of Bacillus are also known for nitrogen fixation and phosphate solubilization (Yadav et al., 2020). Therefore, their presence in the saline soils of Belozem.1 indicates good soil health and fertility. The genus *Sphingomonas* related to Proteobacteria were identified in saline soils of all experimental sites, however, it was highly predominant in Belozem.3. This corroborates the findings that are predominant in saline ecosystems (Menon et al., 2019). This genus thrives in highly saline soil because *of their* robust cell membranes which is stabilized by sphingolipids. *Sphingomonas which are* Gram-negative, and obligate aerobes, possess a remarkable ability to degrade various industrial pollutants and

environmental contaminants which makes them a bioremediation tool (Leys et al., 2004). Some microbes were site specific; for instance, in Belozem.2, *Azotobacter, Candidatus Nitrosophaera* and *Stenotrophomonas* were predominantly observed, indicating unique environmental conditions and nutrient availability that favor their growth and activity.

At the specie level members related to Proteobacteria and Actinobacteriota (*Rhizobium phaseoli, Pseudomonas boreopolis, Arthrobacter crystallopoiestis, Psychrobacillus_psychrodurans, Bacillus_anthracis, Paenibacillus sp JDR-2, Rhodococcus_wratislaviensis among others)* were unique to Belozem.1 (Figure 6). It is possible that the differences in edaphic factors such as pH, soil moisture, EC could have caused the growth of these site-specific microbial species in Belozem.1. The same occurred in Belozem.2 and 3 as each of them had site specific species. This corroborated the findings of (Liu et al., 2024) that soil microorganisms exist in all soils, but their composition and species are mainly determined by environmental factors.

CONCLUSIONS

This study provides an in-depth analysis of the microbiological status of strongly saline soils in Belozem, Bulgaria, combining conventional microbiological assays with 16S rRNA sequencing. The findings revealed that high soil salinity coupled with high soil moisture, and low pH exert a pronounced negative effect on microbial respiration, enzymatic activity, and community diversity. Dominant phyla such as Firmicutes, Proteobacteria, and Actinobacteriota were identified, highlighting their resilience and ecological importance in high-salinity conditions. Additionally, this study found significant variations in microbial diversity and richness across the sampling sites, with Belozem.3 and 2 exhibiting the highest microbial

community structure based on the Shannon diversity index and Belozem.1 showing a lower microbial composition based on the Chao1 index. This diversity indicates that while microbial communities in saline soils share common characteristics, they also exhibit site-specific adaptations that contribute to their overall resilience and ecological function. The presence of atypical saline soil phyla such as Gemmatimonadota and Verrucomicrobiota suggests that microbial communities are capable of evolving and thriving in challenging conditions.

These results have important implications for soil restoration and agricultural productivity in salt-affected regions. Management practices that reduce salinity and improve soil structure such as better drainage, organic amendments, and crop rotation could help rehabilitate microbial function and enhance soil fertility.

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CRediT AUTHORSHIP CONTRIBUTION STATEMENT

Author Contributions: Conceptualization, M.P.,S.S; methodology, G.A., V.P; formal analysis, G.A.,V.P, M.P.; investigation, G.A., V.P, M.P.; resources, S.S., M.P.; data curation, S.S., M.P.; writing - original draft preparation, M.P.; writing - review and editing, S.S., M.P., O.D.; supervision, M.P.; funding acquisition, M.P., S.S. All authors have read and agreed to the published version of the manuscript.

DECLARATION OF COMPETING INTEREST

The authors declare no conflicts of interest.

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526 **ETHICS AND PERMIT APPROVALS**

527 The authors declared neither the lack of any ethical or financial issues nor needs of permissions.

528

529 **DATA AVAILABILITY STATEMENT**

530 The raw sequence data have been deposited in the NCBI Sequence Read Archive (SRA) under
531 BioProject accession number PRJNA1120469 (Belozem saline soils microbiome). The SRA
532 submission ID is SUB14508197, and the dataset includes three biosamples collected on June 5,
533 2024. Processed data are publicly accessible through the SRA portal.

534

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538 MOB.

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540 **AI USE STATEMENT**

541 The authors declare that they did not use artificial intelligence tools in developing this
542 manuscript.

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SAŽETAK

Mikrobna struktura, raznolikost i funkcija u zaslanjenim tlima Belozema, Bugarska: metagenomska analiza i procjena enzimske aktivnosti

Zaslanjena tla predstavljaju značajan izazov za poljoprivrednu produktivnost, osobito u područjima poput Belozema u Bugarskoj, gdje je zaslanjivanje posljedica prirodnih procesa, ali i antropogenih utjecaja. Ova studija istražuje mikrobno stanje izrazito zaslanjenih tala primjenom kombinacije klasičnih mikrobioloških metoda i metagenomskog sekvenciranja. Respiracija tla (SR), supstratom inducirana respiracija (SIR), broj kolonija (CFU) i enzimske aktivnosti (β-glukozidaza i dehidrogenaza) procijenjeni su uz primjenu visokopropusnog sekvenciranja gena 16S rRNA. Rezultati pokazuju da povećana zaslanjenost tla i gravimetrijski sadržaj vode negativno utječu na mikrobnu respiraciju i raznolikost. Mikrobnom zajednicom dominirali su halotolerantni taksoni, uključujući Actinobacteriota, Proteobacteria i Firmicutes. Respiracija tla bila je značajno povezana s vlagom, dok su vrijednosti SIR-a ukazale na visoku razinu mikrobne dormantnosti pod stresom uzrokovanim zaslanjenošću. Analize enzimske aktivnosti pokazale su smanjenu metaboličku aktivnost u uvjetima visoke koncentracije soli, osobito u tlima s najnižim pH vrijednostima i najvišim vrijednostima električne vodljivosti (EC). Metagenomska analiza otkrila je varijacije u alfa i beta raznolikosti među trima tipovima tla, što odražava promjene u strukturi i funkciji mikrobnih zajednica uzrokovane zaslanjenošću. Ovi rezultati naglašavaju ekološke posljedice zaslanjenosti na mikrobnu dinamiku tla te ukazuju na to da metagenomski pristupi mogu pružiti vrijedne uvide za upravljanje ekosustavima zahvaćenima zaslanjenjem.

Ključne riječi: bakterijska mikrobiota tla, respiracija tla, enzimi, halotolerantne bakterije, sekvenciranje 16S rRNA

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758

759 **Table 1.** Basic physical properties of Belozem (pH = power of Hydrogen; EC = Electrical
760 Conductivity; GWC = Gravimetric water content; pH, EC and GWC were significantly different
761 $p<0.005$)

Samples	pH	EC (dS m^{-1})		GWC (g/g)	Land use	Crop cultivated
Belozem.1	6.12[a]	13.56[a]	Strongly saline	58.81[a]	Irrigated field	Rice
Belozem.2	6.88[b]	11.32[b]	Strongly saline	23.99[b]	Limed field	Mixed
Belozem.3	7.23[c]	9.54[c]	Strongly saline	29.89[b]	Old farm	Mixed

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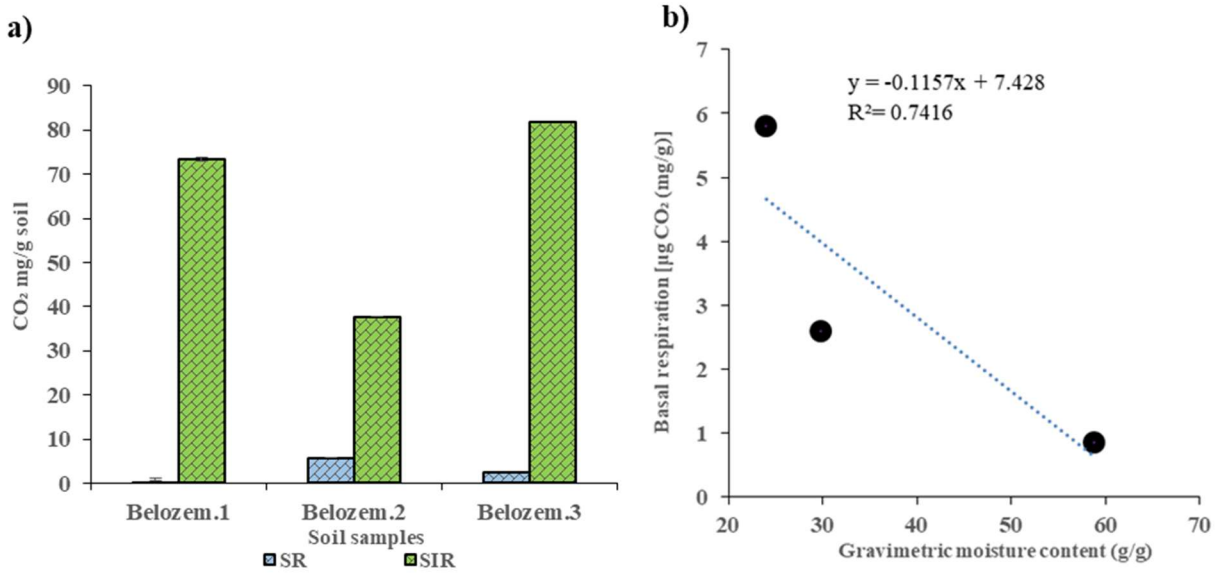

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769 **Figure 1.** Soil respiration and its relationship with soil moisture. **a:** Soil respiration and Soil
770 induced respiration; **b:** relationship between soil respiration and moisture. Error bars represent
771 standard error of means (n=3).

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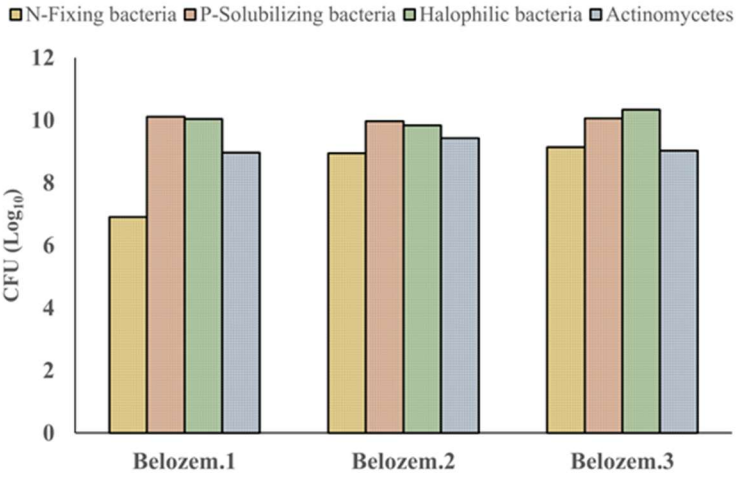

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780 **Figure 2**. Estimation of number of different colony-forming units (cfu) of microbial groups in
781 the sampled soils of Belozem

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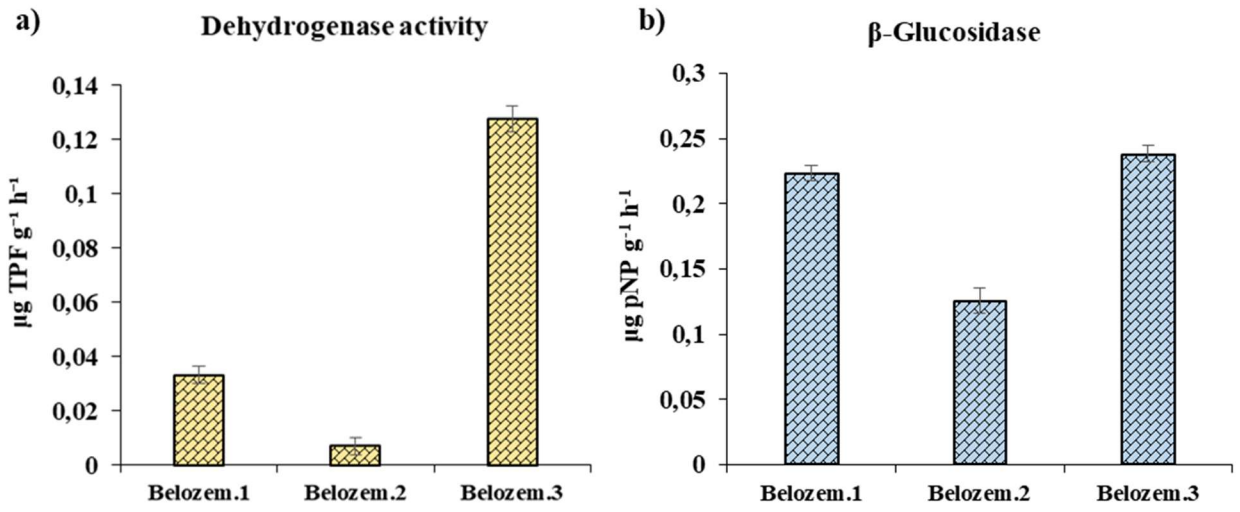

Figure 3. Soil enzymes in saline soils of Belozem. **a:** Dehydrogenase activity; b: β-Glucosidase activity. Error bars represent standard error of means (n=3)

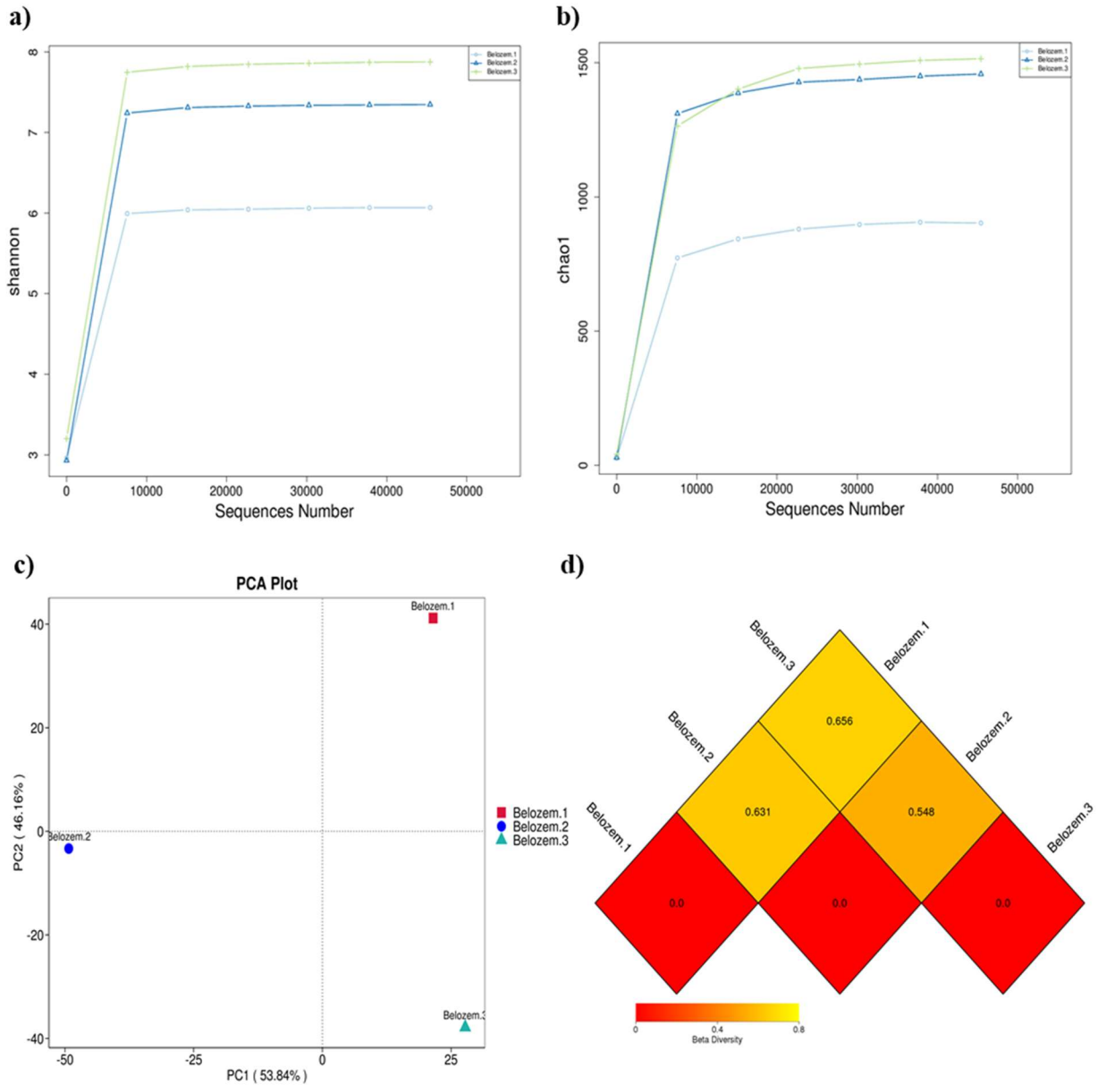

Figure 4. Diversity, Richness and Structure of microorganisms in saline soils of Belozem. **a**: Alpha microbial diversity using Shannon index; **b**: Chao1 degree of richness of microbial communities; **c**: Principal Component Analysis graph showing the structure of microbial communities; **d**: Beta diversity

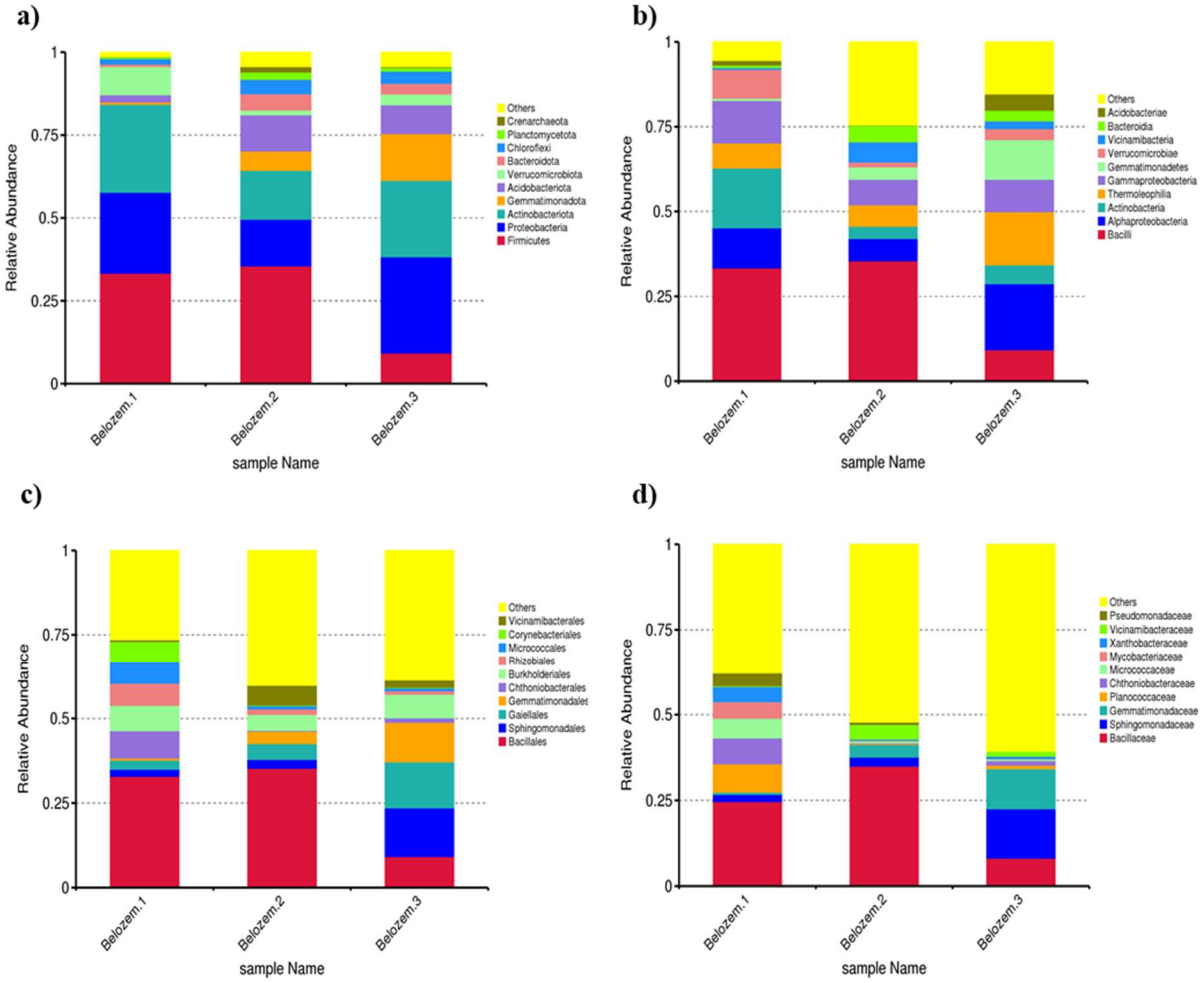

Figure 5: Relative abundance of microbial communities in saline soils of Belozem. **a**: phylum level; **b**: class level; **c**: order level; **d:** family level

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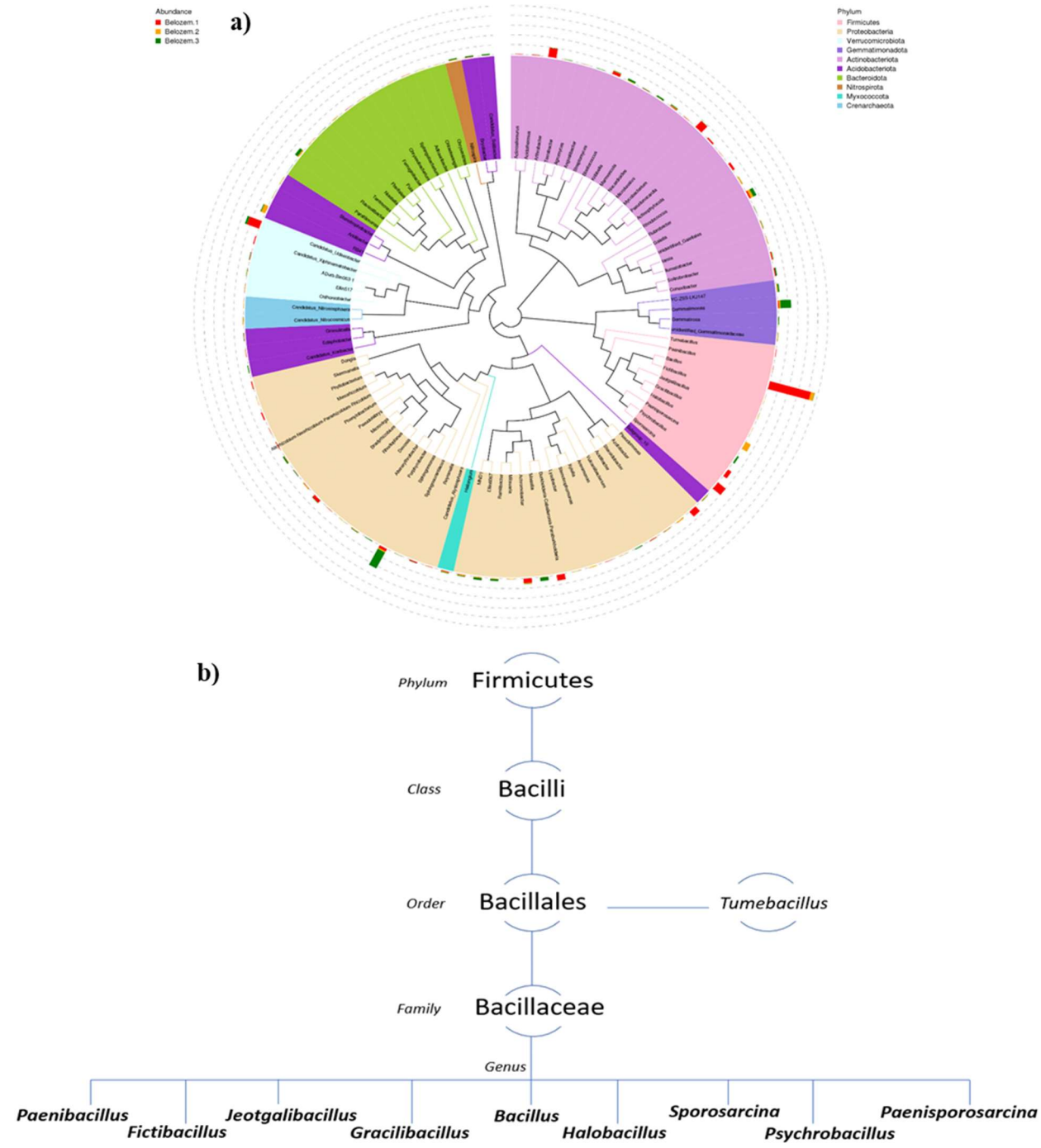

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828 **Figure 6.** Phylogenetic tree of how microorganisms evolved in saline soils of Belozem. a:
829 phylogenetic tree of all microorganisms; b: phylogenetic tree of Firmicutes. The lines in the
830 circle indicates phylum, class, order, family, genus, and species. Bars outside the circle indicates
831 microbes at the sampling sites

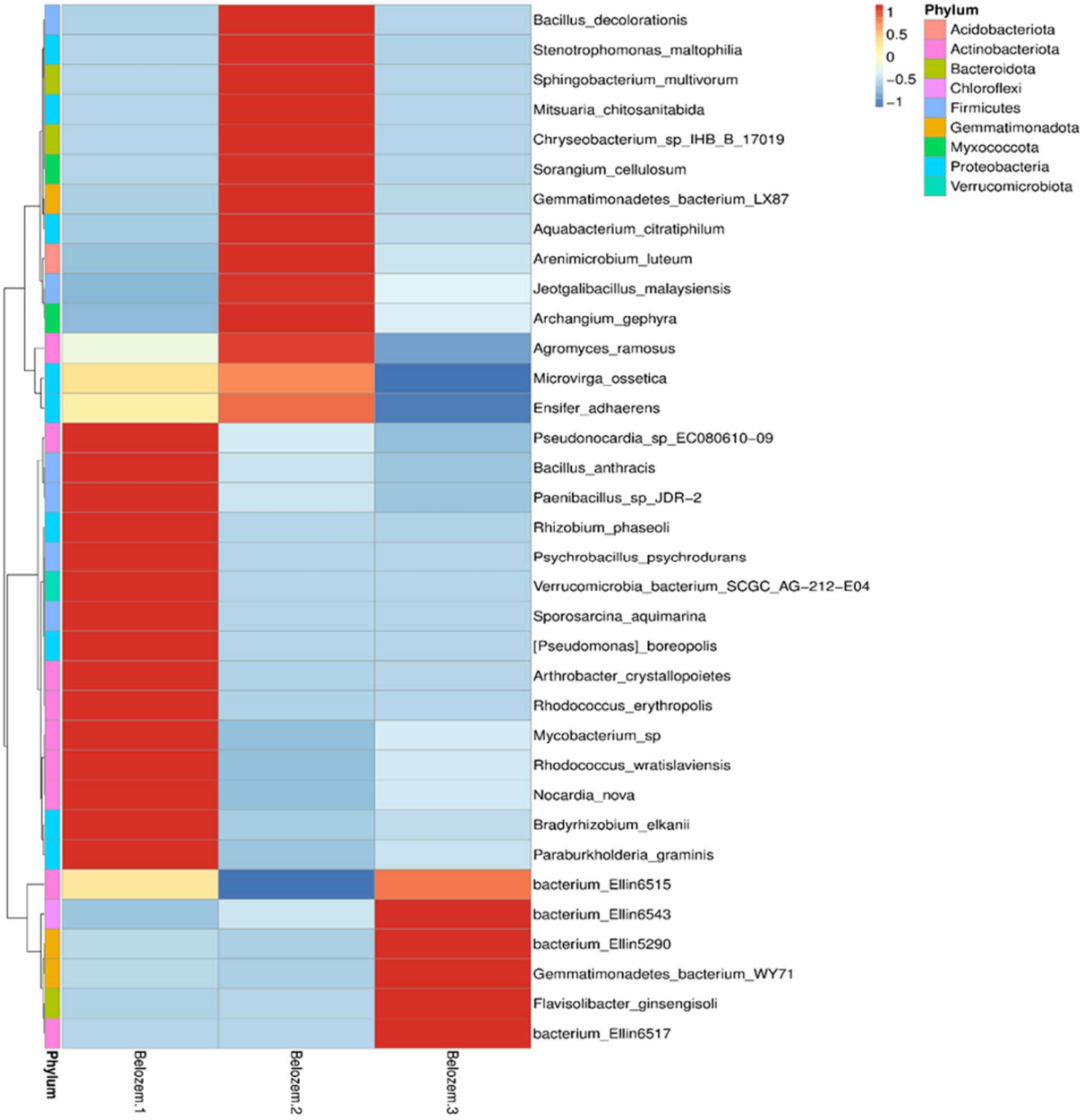

Figure 7. Heat map of the species of microorganisms in saline soils of Belozem