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Divergent responses of growth rate and antioxidative system of ten *Bacillus* strains to acid stresses

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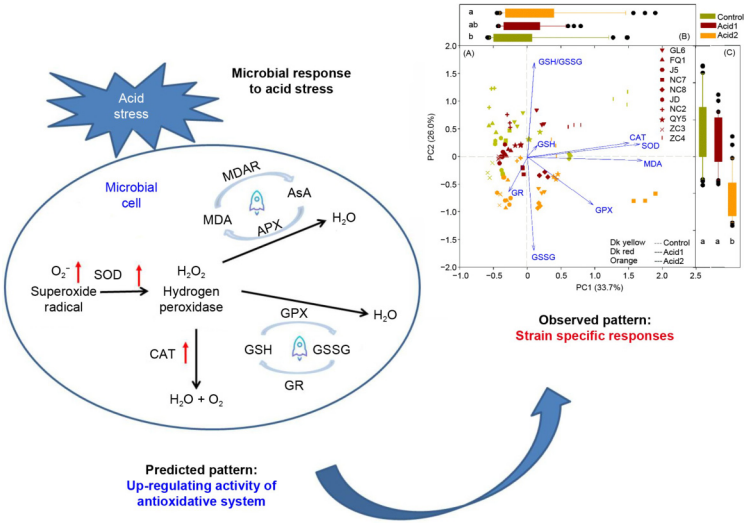
ABSTRACT

• Response of growth rate and antioxidative system of ten *Bacillus* strains to acid stresses was assayed.
• Strong acid treatment significantly decreased the growth rate of the strains.
• Acid stresses increased the GPX activity and GSSG content of the tested strains.
• Divergent changes occurred in ROS and antioxidative system (SOD, CAT, GR, MDA and GSH).

Environmental changes including soil acidification exert obvious stresses on soil ecosystems and influence soil microorganisms. In this study, ten microbial strains were incubated under different acid treatments to investigate responses of microbial growth and antioxidative system to acid stress. All the strains belong to *Bacillus* genus, but exhibit distinct ecological functions. We observed that these microbial strains had obviously different pH tolerance threshold, in spite of the close phylogenetic classification among strains. Acid stresses exerted significant effects on microbial antioxidative system, including superoxide dismutase (SOD), catalase (CAT) and glutathione transferring enzymes (GPX and GR) and reactants (GSH and GSSH), but the effects were strain specific. Furthermore, we found acid stress effects on total variances of the investigated microbial antioxidative system along the first two principal components (PCs). Activities of CAT and SOD contributed substantially to PC1 that reflected obvious acid effects on NC7 and ZC4, and closely related to intracellular malondialdehyde content. The GSSG activities and GSH/GSSG contributed greatly to PC2 that unveiled acid stress effects on most of the microbial strains. Our results highlight substantially heterogeneous responses of microbial strains to acid stress and support that phylogenetic closeness does not imply functional similarity of soil microorganisms under environmental changes.



Keywords soil acidification, microbial strain, acid tolerance capacity, microbial growth, oxidative stress, antioxidative system

1 Introduction

Soil acidification is a natural process that slowly but widely occurs in the soil. With rapid development of technologies and economy of human societies, however, anthropogenic activities such as application of chemical fertilizers and emission of acidic gases have substantially accelerated the process of soil acidification, which influences all kinds of terrestrial ecosystems and biota communities living in these ecosystems. Significant soil acidifications have been reported in diverse terrestrial ecosystems across the world. For instances, Hallbäcken and Tamm (1986) revealed a general decline in soil pH by 0.3−0.9 units after reinvestigation of 90 soil profiles in the forests of south-west Sweden, while Chinese grasslands, croplands and forests are reported to be facing soil acidification in recent decades (Guo et al., 2010; Yang et al., 2012; Yan et al., 2020; Yu et al., 2020). It has been well established that natural processes such as microbial respiration, nitrogen (N) fixation and organic matter mineralization contribute to soil acidifica-

tion relatively slowly, whereas soil acidification processes driven by anthropogenic activities such as application of N fertilizers, atmospheric N deposition and acid rain are often rapid and contribute substantially to declines in acidity of the soil (Markewitz et al., 1998; Raza et al., 2020; Hao et al., 2022).

With widely occurring soil acidification, soil microbial community composition and diversity may have been greatly affected due to high dependence of soil microorganisms on environmental acidity, especially for soil bacterial communities (Fierer, 2017; Bahram et al., 2018; Vetrovsky et al., 2019). Moreover, soil microorganisms are extremely diverse and different microbial groups exert significantly different responses to changes in environmental acidity, which is true for both of bacterial and fungal communities (Rousk et al., 2010). For example, while the relative abundances of alpha-, beta-, gamma-, and delta-*Proteobacteria* and some groups of *Acidobacteria* positively related to soil pH within the range of 4–9, some other groups of *Acidobacteria* were significantly negatively related to soil pH (Rousk et al., 2010). For fungal communities, the relative abundance of *Hypocreales* showed a significantly positive correlation with soil pH, whereas that of *Helotiales* and *Mitosporic basidiomycete* was negatively related to soil pH (Rousk et al., 2010). These observations suggest that the acidity resistance of soil microorganisms is species- or group-specific, which may be attributable to different resistance pathways and capacity of different soil microbial groups (Wei et al., 2023). The altered composition of soil microbial communities could further alter microbial functions, such as litter decomposition and emission of greenhouse gases (Yin et al., 2023; Zhang et al., 2023).

Soil microorganisms are able to maintain intracellular pH homeostasis under a certain extent of environmental acid stresses via diverse pathways (Xu et al., 2020; Chowdhury et al., 2021; Nilsson et al., 2021). When environmental acidity changed, microbes could first try to maintain the intracellular pH level to a certain range, due to shield effect by regulating the fluidity of cell membrane to prevent H^+ from entering the cell, neutralizing effect by promoting the internal metabolic activities to produce alkaline chemicals to consume protons, and pumping effect by regulating the H^+-ATPase activity to extrude H^+ out of cells (Wei et al., 2023). However, exogenous protons will stay in microbial cells due to potential thresholds of these pathways to consume protons, when there is excess H^+ in the environment, and then the intracellular pH condition will be changed (Branson et al., 2021; Wei et al., 2023). Generally, microbial cells will generate reactive oxygen species (ROS, such as superoxide anion and free radicals) in a wide range of metabolic processes and maintain a balance of the ROS production and consumption under natural condition (Aguirre et al., 2005; Winterbourn, 2008). The ROS family may damage cells due to high oxidizing activity and therefore intracellular antioxidative system, including enzymatic or nonenzymatic pathways, plays a role to prevent cell damages by ROS reactions (Aguirre et al., 2005). However, changes in environmental conditions will result in obvious alterations of the ROS level and the pH changes may regulate the production of microbial derived ROS (Winterbourn, 2008; Tullio et al., 2019; Dai et al., 2022). It is often considered that acid stresses will increase aerobic respiration and stimulate the ROS production, therefore inducing oxidative stresses, although the relationship between both has not been completely elucidated (Bruno-Bárcena et al., 2010; Tullio et al., 2019). Consequently, it is expected that regulations would occur in the antioxidative system of microbial cells to reach a new ROS balance under changing environmental conditions. Nevertheless, diverse microbial groups could have different regulation capacity and preferences and therefore the variances in this pathway may contribute to the divergent acid tolerance capacity among soil microbial groups, resulting in changing soil microbial communities under altered pH conditions.

This study was designed to investigate the growth rate of different microbial strains under a series of pH gradient to evidence different acid tolerance capacity among microbial strains and to reveal variances in the microbial antioxidative defense system under different pH conditions. In the present study, ten microbial strains belonging to *Bacillus* genus but having different ecological functions were chosen for incubations and investigations. We hypothesized that the tested microbial strains would exhibit different acid tolerance capacity, considering the species specific nature of microbial communities from multiple aspects, and that the microbial strains would regulate the antioxidative system differently in response to environmental acid stresses; this might potentially contribute to the distinct acid tolerance capacity among microbial strains.

2 Materials and methods

2.1 Microbial strains used in this study

We chose ten microbial strains, of which all belonged to genus *Bacillus*, to use in this study (please see phylogenetic tree of the ten strains in Fig. S1) and all of them were provided by Prof. Yanfei Cai in the *Bacillus* Laboratory of South China Agricultural University, except for one strain (*Bacillus megaterium*, JD) bought from Guangdong Microbial Culture Collection Center. The *Bacillus* strains were chosen based on the observation of our preliminary experiment, in which there was not a clear pattern of microbial response to acid treatments among different genus; using strains in the same genus could at least at the genus scale eliminate the effects of phylogenetic difference. The strains included five

kinds of *Bacillus velezensis* (hereafter named as GL6, NC7, NC8, QY5 and ZC4 in this study), three *Bacillus megaterium* (hereafter named as J5, JD, and ZC3), and two *Bacillus aryabhattai* (hereafter named as FQ1 and NC2), and they had different functions. Therein, GL6, FQ1 and J5 strains were able to produce indole-3-acetic acid to promote the growth of plants; strains NC7 and NC8 were biocontrol strains that were able to control crop pathogens such as rice blast (*Pyricularia oryzae*) and tomato bacter (*Pseudo-monas sollamacearum* (Smith) Smith); strains JD, NC2, and QY5 exerted a function to promote release of soil available phosphorus; and strains ZC3 and ZC4 could promote soil potassium releases. Therefore, these microbial strains are genetically related but functionally divergent. Our preliminary experiment of visual observations by 96-well microplate culture showed that all of these strains could survive and proliferate when the pH of culture medium was above 4.5 or 5.0. Furthermore, these microbial strains were cultured to investigate the growth curve under a series of pH gradients of culture medium as follows.

2.2 Experimental design and strain culture

We first cultured these microbial strains for 48 h under a pH gradient within 2.5−7.0, with an interval of 0.5 pH units. During the incubation period, the optical density at 600 nm (OD_{600}) of the 96-well microplates under different treatments for each microbial strain was periodically read in a Biotek Cytation 5 Image Reader (Agilent Technologies, Inc., Santa Clara, CA, US) to determine the growth condition of microbial strains and used to calculate the growth rate constant of the corresponding microbial strain under different acidity levels (Experiment I).

Furthermore, we set up three acidity levels (control, acid1 and acid2 treatments) to incubate the strains for investigating parameters reflecting the activity of antioxidant system of microbial cells under acid stresses (Experiment II). For all these microbial strains, pH 7.0 was used as the control and pH 5.5 used as the acid1 treatment. However, exact pH value of the acid2 treatment was different based on the observations in Experiment I, because different strains had obviously different pH thresholds for microbial proliferation. Specifically, the acid2 treatment was set at pH 5.0 for strains GL6, JD, and NC2 but at pH 4.5 for the other strains, because the former strains had an obviously lower pH tolerance capacity as revealed in Experiment I. In spite of different pH values, the acid2 treatment might have exerted a comparable acid stress to the tested strains, since the values were close to the corresponding pH threshold of these microbial strains as investigated above and pH 4.5–5.0 were classified as a 'very strong acid' category in the soil acidity level (Chowdhury et al., 2021).

Luria-Bertani (LB) culture medium was used for microbial incubation after adjusting pH to the expected levels by adding HCl solution. The LB medium was chosen for uses in the present study, because it is a widely used bacterial medium with relatively rich nutrients and has been evidenced to be efficient to support bacterial proliferation (Luo et al., 2007; Hassan et al., 2021). Briefly, 2 mL of microbial cells with OD_{600} being 0.4 were pipetted to a sterilized tube containing 20 mL of liquid LB medium at each pH level for incubating at 37°C until OD_{600} reached 0.8. The incubation tubes were then centrifuged at 6000 r min^{-1} under 4°C to collect microbial cells. The obtained microbial cells were transferred to incubate at the corresponding pH condition again for 3 h and then centrifuged (6000 r min^{-1}, 4°C) for 10 min to obtain microbial cells for further analyses.

2.3 Sample analyses

The intracellular ROS was analyzed using a ROS assay kit for bacteria (No. BB-46111, Bestbio Biotech Corp., Shanghai, China), following the assay protocol. This kit consisted of two components, i.e., BBoxiProbe O11 fluorescence probe and probe diluent. In brief, 15 μL of the fluorescence probe were first mixed into 15 mL the probe diluent to make diluted probe solution for the following analysis. Three milliliter of microbial cells were collected as above and cleaned three times using phosphate buffer saline (PBS; pH 7.4). Then, 0.5 mL of diluted probe solution were pipetted and evenly mixed with the cleaned microbial cells to incubate at 37°C for 30 min. After incubation, the microbial cells were collected by centrifugation and cleaned three times by PBS. Finally, 500 μL of PBS were pipetted to resuspend the cleaned microbial cells evenly and 200 μL of samples were transferred into a 96-well plate for analyzing the fluorescence intensity, with the excitation and emission wavelengths at 488 nm and 526 nm, respectively. Intracellular ROS level was indicated by the fluorescence intensity due to the well-established positive relationship between the both.

Microbial enzyme activities and content of intracellular components were assayed after cells were destroyed for 10 min in an ultrasonic processor and the supernate was collected by centrifugation (10000 r min^{-1}, 4°C). Then, glutathione reductase (GR), glutathione peroxidase (GPX), reduced glutathione (GSH), and oxidized glutathione (GSSG) were analyzed using the corresponding assay kit (No. BC1165, BC1195, BC1175 and BC1185, respectively) produced by Beijing Solarbio Science & Technology Co., Ltd (Beijing, China), following the assay proposals (Owens and Belcher, 1965; Alpert and Gilbert, 1985). Moreover, superoxide dismutase (SOD) activity was determined by the photoreduction method of nitrotetrazolium blue chloride (Wu and von Tiedmeann, 2002), and catalase (CAT) activity was tested by the spectrophotometry method at the wavelength of 240 nm, with the H_2O_2 as a key agent (Guan, 1986). The

content of malondialdehyde (MDA) and protein was analyzed by the thiobarbituric acid (TBA) method and coomassie brilliant blue method, respectively.

2.4 Statistics

For each strain, the Spearman correlation was conducted in IBM SPSS Statistics (version 22, IBM Corp., New York, USA) to analyze the significant relationship between the growth rate constant r and medium pH. One-way analysis of variances (ANOVA) was conducted to reveal significant differences among treatments, when both assumptions of data normality and variances homogeneity among groups were met as tested by the Shapiro and Bartlett methods, respectively. Otherwise, non-normally distributed data was analyzed using the Kruskal–Wallis test and Dunn test was then used for multiple comparisons, with the bonferroni method for p value adjustments. The ANOVA and Kruskal–Wallis tests were conducted in R software (version 4.2.1) by the 'PMCMRplus' package. Furthermore, all the intracellular parameters involving in the microbial antioxidative system were pooled to conduct a principal component analysis (PCA) in R by the 'vegan' package, and differences in each of the first two PCs were tested by one-way ANOVA or Kruskal test as mentioned above. Significance level of the statistics was set $p < 0.05$, and $p < 0.01$ indicated extremely significant differences among treatments. All the figures were drawn in Sigmaplot 10.0 (Systat Software Inc., California, USA).

3 Results

3.1 Microbial growth and survival under different pH conditions

Growth curve investigations (Experiment I) showed that three strains (GL6, JD and NC2) could survive and proliferate above pH 5.0, while five strains (FQ1, J5, QY5, ZC3 and ZC4) exhibited growth above pH 4.5 and the other two (NC7 and NC8) above pH 4.0 (Fig. 1). Once environmental pH exceeded the corresponding pH threshold, the tested microbial strains could not proliferate. For each of the microbial

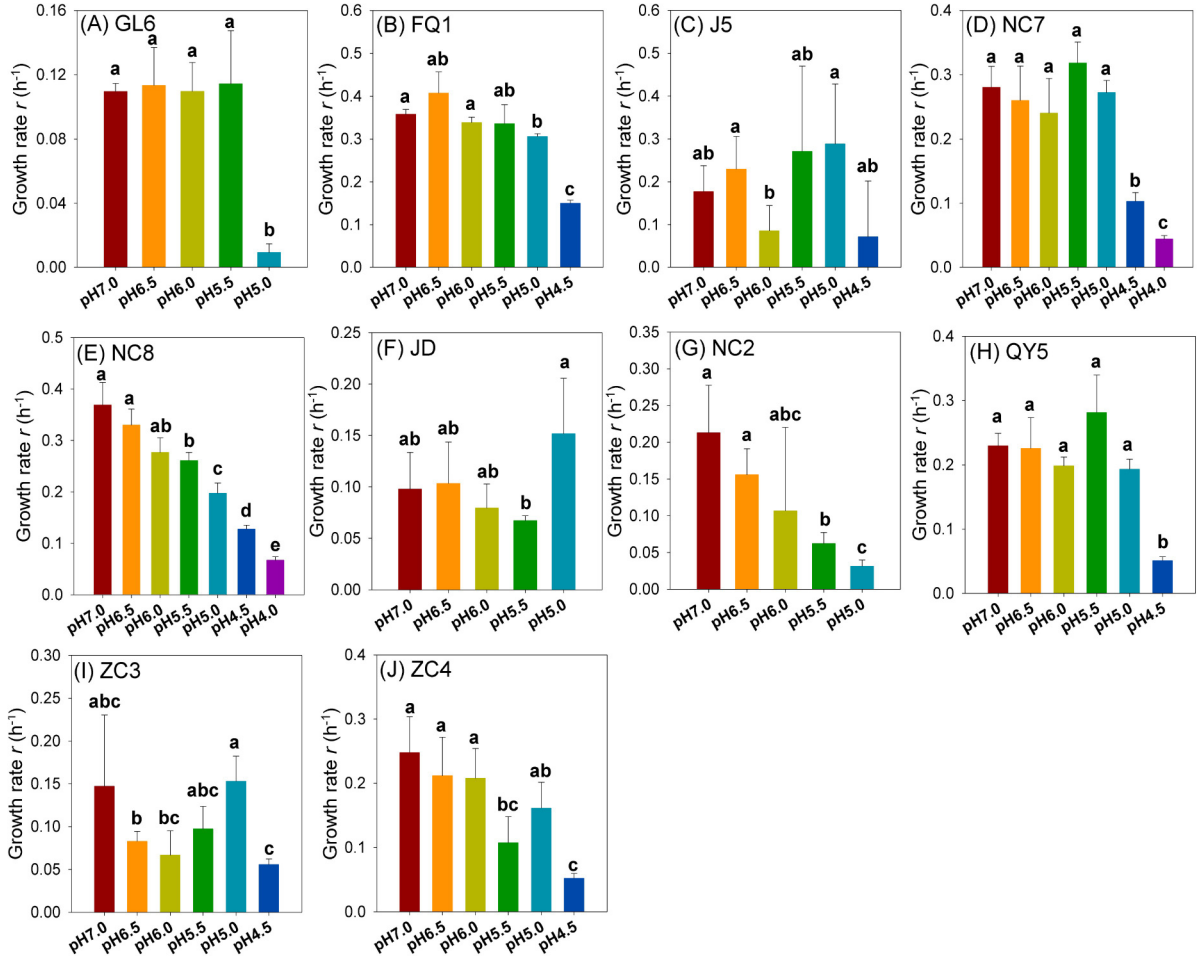


Fig. 1 Growth rate of the ten tested *Bacillus* strains under acid treatments. In each panel, different lowercase letters indicate significant differences among the acid treatments at $p < 0.05$.

strains, the growth rate constant was significantly different among acid treatments ($p < 0.05$ for all), and low pH conditions depressed microbial growth. Relative to the control, extreme acid stress (i.e., pH 5.0 for GL6 and NC2, pH 4.5 for FQ1, QY5 and ZC4, and pH 4.0 for NC7 and NC8) significantly decreased the growth rate of most of microbial strains ($p < 0.05$ for all), except of strains J5, JD and ZC3 ($p > 0.05$, Figs. 1C, 1F and 1I). Moreover, the growth rate constant of four strains (i.e., FQ1, NC8, NC2 and ZC4) was well positively correlated to medium pH ($r > 0.7$, $p < 0.001$), whereas the correlation was not statistically significant for strains J5, JD and ZC3 ($r < 0.2$, $p > 0.05$).

3.2 Intracellular ROS level under different pH conditions

For all of the strains tested in this study, the ROS level was significantly different among acid treatments ($p < 0.05$ for all, Fig. 2). Relative to the control, acid treatments significantly promoted the ROS level in six of the ten tested microbial strains, including GL6, FQ1, NC7, NC8, JD, and QY5 ($p < 0.05$, Fig. 2A, 2B, 2D, 2E, 2F, and 2H), with the most promotion observed for QY5 under acid2 treatment by 161.21% ($p < 0.001$, Fig. 2H). Contrarily, both treatments significantly decreased or tended to decline the ROS level in strains J5, ZC3 and ZC4 ($p < 0.05$, Fig. 2C, 2I and 2J), whereas the ROS level of strain NC2 was not significantly affected by either of the acid treatments ($p > 0.05$, Fig. 2G).

3.3 Antioxidative enzyme activities under different pH conditions

The microbial strains had different initial SOD activity under the control condition, ranging from 0.23 ± 0.026 to 1.87 ± 0.085 U mg^{-1} protein, which was promoted or declined by acid treatments (Table 1). In details, the relatively weak acid treatment significantly increased the SOD activity in strains GL6, FQ1, J5, NC2, and ZC3 ($p < 0.05$), while the strong acid treatment significantly increased the activity only in strain NC2 but significantly decreased the SOD activity in strains FQ1, J5, NC8, and ZC4 ($p < 0.05$). The CAT activity was significantly promoted by acid treatments in strains FQ1, NC7, NC2, QY5, and ZC3 ($p < 0.05$), but significantly

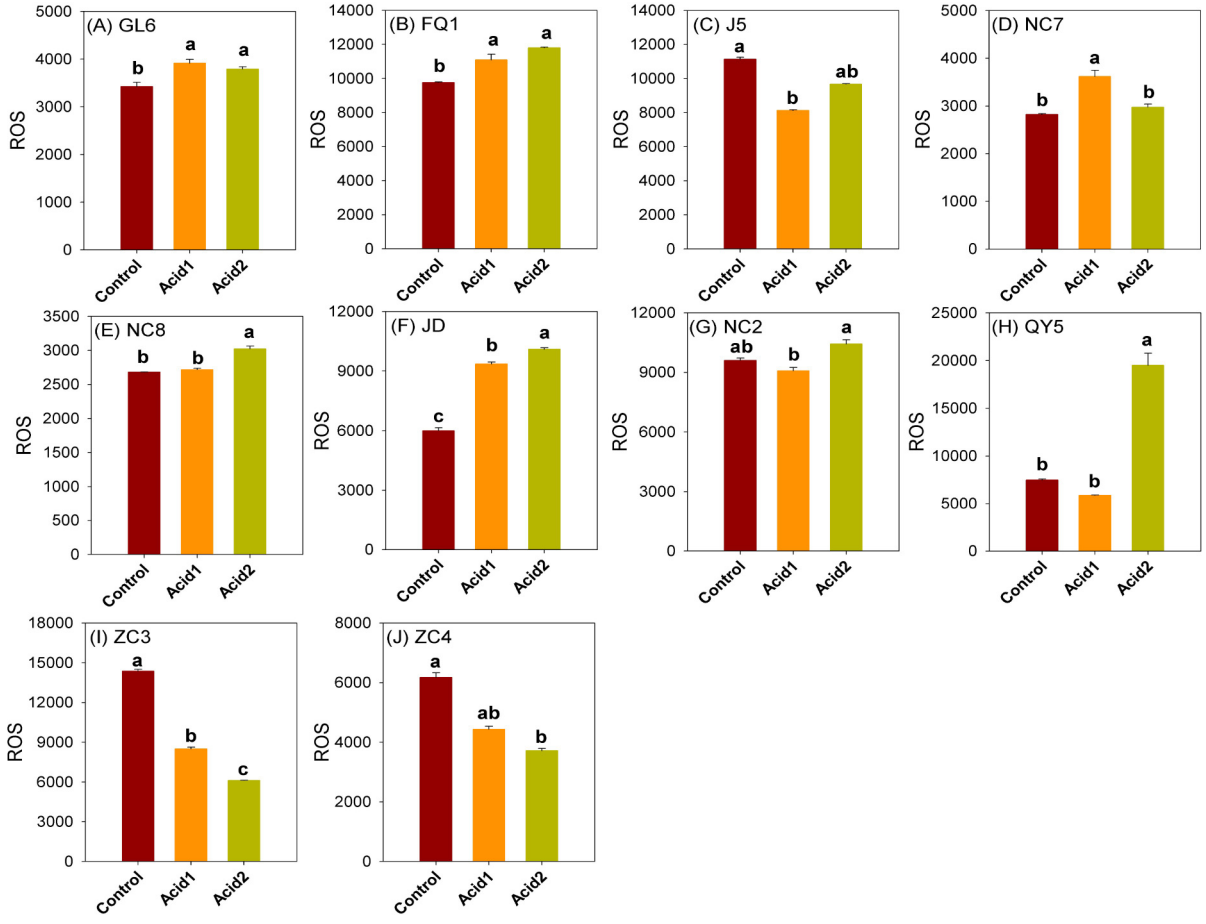


Fig. 2 Reactive oxygen species (ROS) of the ten tested *Bacillus* strains under acid treatments. In the figure, Acid1 represents a relatively weak acid treatment of pH 5.5, while Acid2 represents a very strong acid treatment of pH 5.0 (for strains GL6, JD, and NC2) or 4.5 (for the other strains), with the control of pH 7.0. In each panel, different lowercase letters indicate significant differences among the acid treatments at $p < 0.05$.

Table 1 Effects of acid treatments on the antioxidative enzymes of different microbial strains.

	Treatment	GL6	FQ1	J5	NC7	NC8	JD	NC2	QY5	ZC3	ZC4
SOD	Control	0.63 (0.011)b	0.35 (0.018)b	0.32 (0.023)b	0.83 (0.038)ab	1.59 (0.026)a	0.32 (0.0025)ab	0.23 (0.026)c	1.06 (0.18)ab	0.25 (0.0052)b	1.87 (0.085)a
	Acid1	1.04 (0.0051)a	0.43 (0.025)a	0.44 (0.021)a	0.76 (0.024)b	1.02 (0.11)b	0.31 (0.0058)b	0.50 (0.018)a	0.60 (0.14)b	0.34 (0.010)a	1.22 (0.089)c
	Acid2	0.83 (0.15)ab	0.0030 (0.0020)c	0.22 (0.019)c	1.84 (0.090)a	0.78 (0.049)c	0.39 (0.0027)a	0.44 (0.014)b	1.55 (0.037)a	0.32 (0.0039)ab	1.46 (0.026)b
CAT	Control	1.48 (0.18)a	0.55 (0.026)b	0.86 (0.081)a	0.33 (0.066)b	1.52 (0.10)	0.93 (0.072)a	0.49 (0.11)b	0.51 (0.052)b	0.35 (0.037)b	3.81 (0.51)a
	Acid1	1.31 (0.093)a	0.99 (0.081)a	0.58 (0.13)b	0.25 (0.028)b	1.25 (0.15)	0.33 (0.046)c	1.21 (0.12)a	0.61 (0.038)b	0.52 (0.10)a	1.36 (0.27)b
	Acid2	0.68 (0.15)b	1.23 (0.15)a	0.41 (0.031)b	3.51 (0.58)a	1.25 (0.036)	0.71 (0.082)b	1.31 (0.093)a	0.81 (0.039)a	0.28 (0.023)b	0.60 (0.13)b
GPX	Control	0.18 (0.0015)b	0.23 (0.013)b	0.24 (0.0015)b	0.20 (0.0079)b	0.31 (0.0039)ab	0.22 (0.0084)c	0.22 (0.0030)c	0.28 (0.0020)b	0.25 (0.010)b	0.26 (0.010)b
	Acid1	0.21 (0.019)ab	0.26 (0.0043)a	0.24 (0.00062)ab	0.28 (0.00062)ab	0.28 (0.0065)b	0.29 (0.0046)a	0.23 (0.0015)b	0.27 (0.0047)c	0.25 (0.0042)b	0.31 (0.0053)a
	Acid2	0.24 (0.0013)a	0.25 (0.0011)a	0.28 (0.0063)a	0.32 (0.011)a	0.31 (0.0066)a	0.25 (0.0022)b	0.26 (0.0011)a	0.29 (0.0025)a	0.27 (0.0040)a	0.29 (0.0079)a
GR	Control	0.058 (0.0092)b	0.17 (0.0092)ab	0.093 (0.014)	0.087 (0.0035)b	0.085 (0.0095)b	0.12 (0.042)	0.14 (0.0059)a	0.12 (0.0092)a	0.20 (0.013)a	0.088 (0.0040)ab
	Acid1	0.073 (0.0099)ab	0.16 (0.010)b	0.093 (0.0038)	0.10 (0.0038)ab	0.11 (0.0096)ab	0.13 (0.051)	0.11 (0.0061)b	0.084 (0.0071)b	0.15 (0.023)ab	0.069 (0.0043)b
	Acid2	0.11 (0.025)a	0.19 (0.0060)a	0.075 (0.0038)	0.18 (0.020)a	0.19 (0.0041)a	0.25 (0.072)	0.085 (0.032)ab	0.10 (0.0034)ab	0.080 (0.0069)b	0.11 (0.0047)a

The term Acid1 represents a relatively weak acid treatment of pH 5.5, while Acid2 represents a very strong acid treatment of pH 5.0 (for strains GL6, JD, and NC2) or 4.5 (for the other strains), with the control of pH 7.0. Results are presented as means and the corresponding standard deviations in the brackets. In the table, SOD is the abbreviation of superoxide dismutase, while CAT, GR, GPX stand for catalase, glutathione reductase, and glutathione peroxidase, respectively. For each of the strains, different lowercase letters indicate significant differences among the acid treatments at $p < 0.05$.

reduced in strains GL6, J5, JD, and ZC4 ($p < 0.05$) or tended to reduce in strain NC8 ($p = 0.065$).

Acid treatments consistently and significantly increased the GPX activity for all the tested microbial strains ($p < 0.05$), except strain NC8 that had significantly higher GPX under pH 4.5 than pH 5.5 treatments ($p = 0.0497$). Relative to the control, strong acid treatment significantly promoted the GPX activity in strains GL6, J5, NC7, QY5, and ZC3 ($p < 0.05$ for all), while both treatments significantly increased the activity in strains FQ1, JD, NC2, and ZC4 ($p < 0.05$ for all). The GR activity showed divergent responses to acid treatments among different microbial strains. In brief, strong acid treatment significantly increased the GR activity in strains GL6, NC7, and NC8, but significantly reduced it in strain ZC3 ($p < 0.05$ for all). Weak acid treatment significantly reduced the GR activity in strains NC2 and QY5 ($p < 0.05$ for both). However, either of the acid treatments significantly altered the GR activity in strains FQ1, J5, JD, and ZC4 relative to the control ($p > 0.05$ for all, Table 1).

3.4 Intracellular components under different pH conditions

The tested microbial strains showed significantly different intracellular MDA content in the control condition, with the strain ZC4 being the highest but NC2 the lowest (Table 2). The intracellular MDA content was significantly promoted especially by strong acid treatment in strains GL6, FQ1, J5, NC2, JD, NC2, and QY5 ($p < 0.05$), not significantly changed by either of the two treatment levels in strains NC8 and ZC3 ($p > 0.05$), but significantly decreased in ZC4 by both acid treatments ($p < 0.05$), relative to the control (Table 2). Moreover, the relatively weak acid treatment of pH 5.5 did not significantly alter the MDA content of strains FQ1, J5, NC7, NC8, JD, QY5, and ZC3 ($p > 0.05$).

The GSH content was significantly changed by acid treatments in seven of the ten tested strains, i.e., FQ1, J5, NC7, NC8, JD, NC2, and ZC4 ($p < 0.05$, Table 2). Relative to the control, both acid treatments significantly promoted the GSH content for strains J5, NC7, JD, and NC2 ($p < 0.05$ for all), while strong rather weak acid treatment significantly increased it for strains FQ1, NC8, and ZC4 ($p < 0.05$). On the contrary, both acid treatments significantly declined the GSH content in strain NC2. The GSH content was not significantly altered by either of the two treatments for strains GL6 and QY5 ($p > 0.05$ for both).

For each of the tested strains, the GSSG content was substantially lower than the GSH content, and the treatments consistently increased the GSSG content for all the strains, marginally significantly (GL6: $p = 0.051$; JD: $p = 0.065$) or statistically significantly ($p < 0.05$ for all the others; Table 2).

Table 2 Effects of acid treatments on the intracellular components of different microbial strains.

	Treatment	GL6	FQ1	J5	NC7	NC8	JD	NC2	QY5	ZC3	ZC4
MDA	Control	0.16 (0.0086)c	0.062 (0.0031)b	0.089 (0.013)b	0.15 (0.016)b	0.20 (0.017)	0.065 (0.0033)b	0.056 (0.0024)c	0.15 (0.0095)b	0.058 (0.0028)ab	0.45 (0.030)a
	Acid1	0.25 (0.00062)b	0.075 (0.0069)b	0.074 (0.0057)b	0.16 (0.0015)b	0.19 (0.014)	0.067 (0.0031)ab	0.069 (0.0059)b	0.12 (0.0093)b	0.065 (0.0038)a	0.33 (0.019)b
	Acid2	0.36 (0.0040)a	0.13 (0.0098)a	0.12 (0.012)a	0.62 (0.033)a	0.14 (0.0013)	0.074 (0.0038)a	0.099 (0.0022)a	0.20 (0.018)a	0.053 (0.00076)b	0.22 (0.013)c
GSH	Control	4.60 (0.50)	4.16 (0.12)b	3.38 (0.22)c	3.54 (0.31)c	3.72 (0.072)b	3.97 (0.12)c	5.79 (0.84)a	5.22 (0.21)	3.87 (0.18)ab	3.92 (0.20)b
	Acid1	5.23 (0.52)	4.59 (0.15)ab	3.83 (0.048)b	4.21 (0.18)b	3.56 (0.12)b	4.42 (0.17)b	4.05 (0.22)b	4.85 (0.12)	4.46 (0.35)a	4.46 (0.14)ab
	Acid2	3.93 (0.50)	5.87 (1.09)a	4.41 (0.18)a	5.08 (0.099)a	4.37 (0.11)a	5.51 (0.19)a	4.32 (0.16)b	5.33 (0.38)	3.47 (0.19)b	5.25 (0.58)a
GSSG	Control	0.37 (0.0084)	0.27 (0.056)b	0.57 (0.028)b	0.36 (0.045)c	0.39 (0.030)c	0.36 (0.015)	0.26 (0.074)b	0.36 (0.015)b	0.46 (0.12)b	0.21 (0.028)b
	Acid1	0.37 (0.0087)	0.41 (0.054)b	0.46 (0.081)b	0.51 (0.039)b	0.52 (0.047)b	0.36 (0.017)	0.27 (0.071)b	0.41 (0.015)b	0.45 (0.059)b	0.27 (0.027)b
	Acid2	0.77 (0.046)	0.85 (0.13)a	0.86 (0.056)a	0.79 (0.041)a	0.74 (0.055)a	0.63 (0.016)	0.49 (0.022)a	0.67 (0.030)a	0.76 (0.098)a	0.42 (0.041)a
GSH/GSSG	Control	12.36 (1.35)a	15.28 (0.46)a	5.97 (0.39)b	9.75 (0.85)a	9.67 (0.19)a	10.96 (0.33)b	22.62 (3.28)a	14.53 (0.59)a	8.37 (0.40)b	18.43 (0.96)a
	Acid1	14.08 (1.40)a	11.33 (0.36)ab	8.42 (0.11)a	8.28 (0.36)b	6.81 (0.24)b	12.12 (0.48)a	14.82 (0.81)b	11.88 (0.29)b	10.02 (0.79)a	16.30 (0.50)ab
	Acid2	5.14 (0.65)b	6.93 (1.29)b	5.16 (0.21)c	6.41 (0.12)c	5.94 (0.15)c	8.82 (0.30)c	8.85 (0.34)c	7.98 (0.56)c	4.56 (0.25)c	12.42 (1.37)b

The term Acid1 represents a relatively weak acid treatment of pH 5.5, while Acid2 represents a very strong acid treatment of pH 5.0 (for strains GL6, JD, and NC2) or 4.5 (for the other strains), with the control of pH 7.0. Results are presented as means and the corresponding standard deviations in the brackets. In the table, MDA, GSH and GSSG stand for malondialdehyde, reduced glutathione, and oxidized glutathione, respectively. For each of the strains, different lowercase letters indicate significant differences among the acid treatments at $p < 0.05$.

Combining the two parameters together, we found that strong acid treatment consistently and significantly declined the GSH/GSSG ratio in all the tested strains ($p < 0.05$), while the relatively weak acid treatment did not significantly change the ratio in strains GL6, FQ1, and ZC4 ($p > 0.05$), but significantly increased it in strains J5, JD, and ZC3 ($p < 0.05$, Table 2).

3.5 The total variance in the tested microbial properties under different pH conditions

The results of PCA presented the total variance in the intracellular components and antioxidative system of microbial strains in this study and the first two principal components (PCs) together explained 59.7% of the total variance (Fig. 3). The parameters including CAT, SOD and MDA contributed substantially to PC1 component, while GSSG and GSH/GSSG ratio greatly contributed to PC2 component (Fig. 3A). Under the control condition (as indicated by dark yellow), we could observe obvious isolations of the tested microbial strains in the two-dimension coordinate system consisting of the first two PCs (Fig. 3A). Acid treatments, especially the strong acid treatment (indicated by the orange labels), appeared to affect the microbial properties (Fig. 3A). Further analyses revealed that there were significantly differences in PC1 and PC2 scores among treatments ($p < 0.05$ for both, Figs. 3B and 3C), indicating that acid treatments significantly altered the intracellular components and oxidative system of these microbial strains.

4 Discussion

As expected, the ten microbial strains had obviously different acid tolerance capacity, because they survived and proliferated at different lower pH threshold ranging from 4.0 to 5.0 (Fig. 1). The two strains NC7 and NC8 exhibited obviously the highest acid tolerance capacity, while strains GL6, JD and NC2 had the lowest capacity to resist to acid stress. The observation suggests that microbial acid tolerance capacity is strain specific and may vary among microbial strains even when they are phylogenetically or functionally related (Wei et al., 2023). As summarized in previous studies, microorganisms are able to maintain the intracellular pH homeostasis within certain pH ranges by regulating cell physiological condition (Lund et al., 2014; Wu et al., 2014; Chowdhury et al., 2021). Microbial individuals could down-regulate cell membrane fluidity to prevent influx of proton by shifting the composition of membrane fatty acids (Quivey Jr. et al., 2000; Yoon et al., 2015), upregulate metabolic activities

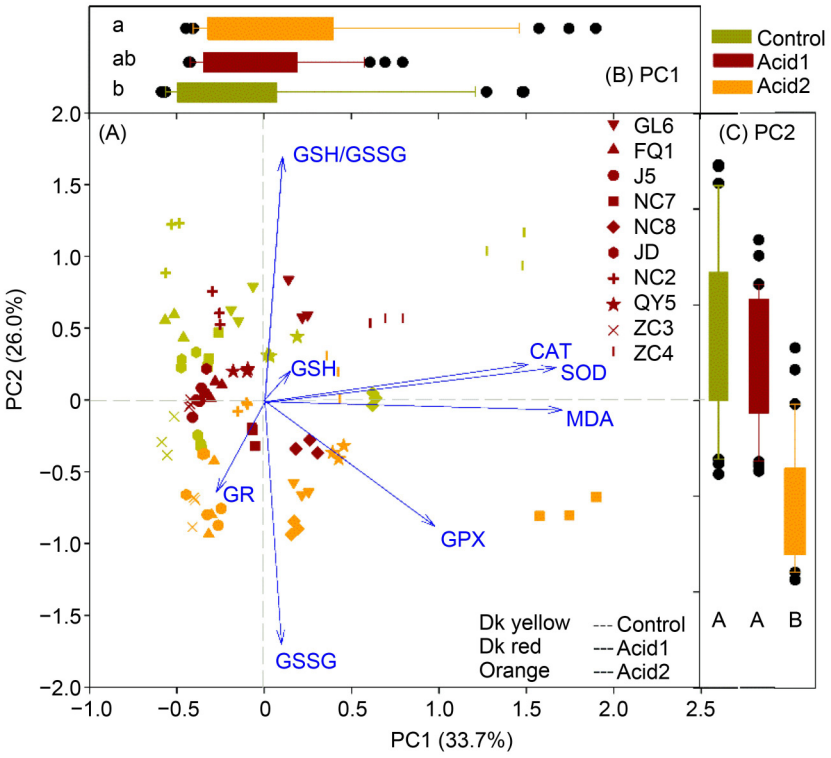


Fig. 3 Result visualization of principal component analysis on the antioxidative system under acid treatments. In the figure, Acid1 represents a relatively weak acid treatment of pH 5.5, while acid2 represents a very strong acid treatment of pH 5.0 (for strains GL6, JD, and NC2) or 4.5 (for the other strains), with the control of pH 7.0. In panel A, different shapes indicate different microbial strains, while different colors indicate the acid treatments in all the three panels. In each of panels B and C, different lowercase or upper letters indicate significant differences among the acid treatments at $p < 0.05$. The abbreviations SOD, CAT, GR, GPX, MDA, GSH and GSSG stand for superoxide dismutase, catalase, glutathione reductase, glutathione peroxidase, malondialdehyde, reduced glutathione, and oxidized glutathione, respectively.

to produce more alkaline components or promote amino acid metabolisms to consume proton (Lund et al., 2014; Nilsson et al., 2021), and pump excess proton out of microbial cells due to enhancement of proton pumps (Tullio et al., 2019; Guan and Liu, 2020); these pathways contribute to the acid tolerance capacity of microbial cells and distinct capacity to regulate these pathways may result in different acid tolerance thresholds among strains (Wei et al., 2023). Interestingly, we observed that some of the microbial strains responded to the pH gradient in an approximately linear way (e.g., NC8 [Fig. 1E] and NC2 [Fig. 1G]), whereas some others responded abruptly (e.g., GL6 [Fig. 1A] and QY5 [Fig. 1H]), indicating that the potential acid tolerance mechanisms are likely different.

In this study, acid treatments induced significant alterations in the ROS level that were observed to increase in most of the tested microbial strains as expected (Fig. 2). Although potential mechanisms have not been completely elucidated, acid stresses are often considered to associate with oxidative stresses that derive from higher intracellular ROS levels (Bruno-Bárcena et al., 2010; Tullio et al., 2019), which is supported by the observations of higher ROS levels under acid treatments in this study. The ROS components are mainly generated from respiratory chain and aerobic respiration contributes greatly to the production of ROS (Aguirre et al., 2005). Acid stresses could induce aerobic respiration and therefore promote the ROS level in microbial cells (Tullio et al., 2019), resulting in increased ROS as observed in this study. However, we also observed exceptions in three microbial strains, i.e., J5, ZC3 and ZC4 (Figs. 2C, 2I and 2J), suggesting that acid stresses do not necessarily equal to oxidative stresses. The intracellular ROS level is determined by the tradeoff of ROS production and consumption in microbial cells, and the tradeoff could be changed under environmental changes such as acidified conditions. Although potential mechanisms remain unclear, our results of the divergent regulations among the microbial strains indicate that acid stresses would not exert fixed effects on the production and consumption processes of ROS components and therefore will not consistently increase or decrease the intracellular ROS level. Consequently, this may result in different oxidative stresses across diverse microbial strains.

Microbial cells have developed diverse enzymatic and nonenzymatic pathways to scavenge ROS components and prevent cells from oxidative damages caused by intracellular ROS reactions (Jamieson, 1998; Aguirre et al., 2005;

Winterbourn, 2008). It is widely known that the SOD is the first line of defense against ROS, due to its capacity to scavenge superoxide radicals that widely exist in different compartments of a cell and will over-produce under abiotic stresses (Wingsle and Hallgren, 1993; Alscher et al., 2002). In association with the altered ROS level under acid treatments, the SOD activity was promoted in several microbial strains tested in our study, which indicates a potentially higher activity to scavenge ROS and prevent cell damages in these strains such as GL6 and NC2 (Table 1). However, we found that not all of the strains upregulated the SOD activity when the intracellular ROS level was increased, but regretfully the reasons are not revealed in the present study. The SOD family consists of different metalloenzymes, i.e., iron SOD, manganese SOD and coper-zinc SOD, which locate at different compartments of a cell and may respond differently to changing environmental conditions (Alscher et al., 2002). The SOD activity is determined by the variances in the three groups of enzymes under acid treatments and further differentiation of enzyme classifications are required to reveal the effects of acid stresses on SOD activity in future studies.

The SOD is able to dismutate superoxide radicals to generate H_2O_2 that will be further decomposed via multiple pathways, including enzyme (CAT and GPX) catalyzing ways and abiotic Fenton reaction (Winterbourn, 2008; Gill and Tuteja, 2010). The CAT catalyzing pathway is important to the breakdown of H_2O_2 to water and oxygen (Mittler, 2002). In the present study, we observed that the CAT activity was significantly altered in response to acid treatments (Table 1). The PCA visualization showed a close relationship between SOD and CAT (as reflected by the small angle between both; Fig. 3A), supporting the synergetic effect of both when facing abiotic environmental stresses such as acidified conditions. Moreover, both SOD and CAT contributed substantially to the acid induced variances of microbial strains along with PC1 (Fig. 3A), which could to a considerable extent explain the variances of microbial strains (especially for NC7 and ZC4) under acid treatments. Associated with the shifts in SOD and CAT activities, intracellular MDA content was significantly increased in most of the tested microbial strains (Table 2). This indicates that in spite of increased antioxidative enzyme activities such as SOD and CAT, microbial cells experienced more severe lipid peroxidation under acid stress (Joshi et al., 2011) and different magnitude of lipid peroxidation could also contribute to distinct acid tolerance capacity of these microbial strains. Such obvious shifts resulted in the significant differences in PC1 scores among treatments (Fig. 3B).

For most of the tested microbial strains, however, the acid induced variance in microbial physiology was clearly reflected by PC2, since there were clear isolations among treatments along with PC2 (e.g., FQ1 and NC2; Fig. 3A) that explained the significant differences in PC2 among treatments (Fig. 3C). Such regulations are able to associate with changes in the reversible GPX catalyzing pathways, in which GSH, GSSG and two enzymes including GPX and GR involve (Mittler, 2002; Wang et al., 2010). In this study, we observed consistent increases in the GPX activity for all the microbial strains under acid treatments than under the control, with an only exception of NC8 for which the difference was not statistically significant (Table 1). This scenario suggests that the GPX catalyzing pathway may be a key player consistently to these *Bacillus* strains in response to acid stress. The GPX family consists of diverse isozymes that scavenge oxidative stress by reducing H_2O_2 and hydroperoxides in cells (Gill and Tuteja, 2010). A series of proteins and genes regulate the GPX activity (Noctor et al., 2002), and therefore may have played an important role in mitigating acid induced oxidative stress to the tested microbial strains. However, the GR activity was not changed consistently across all the microbial strains (Table 1), indicating the balance of GPX and GR catalyzing processes might have been shifted under acid treatments but the shift is obviously various across microbial strains. This shifted balance may be a microbial strategy to survive under acid stress and result in differences in acid tolerance capacity of the tested microbial strains (Fig. 1).

Associated with changes in the GPX activity, the GSSG content was significantly promoted by acid treatments (especially the strong acid treatment) for all the tested microbial strains (Table 2), which contributed greatly to PC2 of the PCA visualization (Fig. 3A). The GSSG can be produced by multiple reactions in which the GSH involves and the balance between GSH and GSSG is of importance to maintain redox condition within cells (Gill and Tuteja, 2010). Our observations suggest that microorganisms could regulate the intracellular GPX cycle to mitigate oxidative stress under acid conditions. Resultantly, the ratio of GSH/GSSG was significantly decreased by acid (especially strong acid) treatments (Table 2), indicating that acid stress stimulated the process of reductive GSH transforming into GSSG via GPX catalyzation. This catalyzing pathway contributes substantially to intracellular ROS scavenging, as demonstrated in previous studies (Mittler, 2002; Aguirre et al., 2005; Gill and Tuteja, 2010), as well as in the present study. Moreover, our results suggest that relative to GSH content, GSH/GSSG ratio or GSSG content contributed more to ROS scavenge in these tested microbial strains under acid stress, which is in line with plant cells as reported previously (Komives et al., 1998; Wang et al., 2010).

Notably, we observed that the growth rate and ROS system of the tested microbial strains responded differently to acid treatments (Figs. 1−3 and Tables 1−2), although all of the strains belonged to the *Bacillus* genus that were

phylogenetically related (Fig. S1). The pattern is out of our expectation, but it highlights the fact that phylogenetic relationship won't necessarily determine the response of these *Bacillus* strains to changes in environmental acid conditions. Previous studies suggest that the soil microbial communities could respond differently to the changes in soil pH at the phylum level (Rousk et al., 2010; Puissant et al., 2019). Further, our observations indicate that the response of soil microorganisms to changing environmental acidity may be strain specific. This amplifies the uncertainty for the prediction of soil microbial dynamics under environmental changes.

Soil microbial communities drive multiple soil ecological processes such as mineralization and stabilization of soil organic carbon (SOC) and N cycling, as well as the ecosystem stability under climate change (Delgado-Baquerizo et al., 2017; Crowther et al., 2019; Yin et al., 2023). Due to soil acidity induced changes in the soil microbial community composition, the soil ecological functions driven by soil microbial communities may be also affected and consequently influence ecosystem stability, especially under rapidly changing environmental conditions (Puissant et al., 2019; Zhou et al., 2020). This study highlights divergent responses of the acid tolerance capacity and antioxidative system in different microbial strains with exposures to acid stress. The differences as observed in the present study provide insights to reveal the community-level changes in soil microbial communities under environmental changes especially with soil acidification; under acidifying environmental conditions, diverse soil microbial strains exhibit obviously various acid tolerance capacity that may derive from different capacity to regulate their cell physiology for dealing with the occurring environmental stresses. Therefore, shifts will occur in the composition of soil microbial communities under changing environments, and the ecological functions driven by soil microorganisms will also change correspondingly.

In spite of the observations, however, we recognize that an obvious limitation exists to extend these results in field studies, because the results are obtained in a pure culture experiment by using nutrient medium that is far different from the real soil environment. Such a simplification provides us an easier way to understand the underlying mechanisms of these tested microbial strains in response to acidifying environment, but the results could, at least in part, be different from field investigations (Jensen et al., 1998), considering the extremely high soil heterogeneity that could influence microbial response to environmental changes (Nunan et al., 2020). Further studies to verify the conclusions in plant–soil systems remain needed, when more advanced techniques are proposed to isolate and purify a given microbial strain targeted in a study with an aim to precisely reveal effects of environmental changes on a specific microbial species or strains at the cellular level.

5 Conclusions

In this study, we incubated ten *Bacillus* strains that were phylogenetically similar but functionally different under acid treatment conditions for investigations of the growth curve and intracellular antioxidative system. It was observed that although all the microbial strains belong to the same genus, these microbial strains exhibited obviously different acid tolerance capacity, since they might survive and proliferate under different extreme pH conditions ranging from 4.0 to 5.0. Moreover, different microbial strains showed different capacities to regulate the intracellular antioxidative system (including SOD, CAT and the GPX catalyzing pathway) for dealing with the oxidative stress induced by acid treatments. Such distinct shifts in the intracellular antioxidative system could at least in part contribute to different acid tolerance capacity of the investigated *Bacillus* strains, supporting that various microbial strain level response may give rise to the changes in soil microbial community composition in response to environmental changes such as acidifying conditions.

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Authors' contribution

HW, XS and JZ conceived the experiment. XS, JC, ZL, and SC finished incubation and analyzed samples to collect data, analyzed and visualized data. XS and HW wrote the draft. JZ reviewed the manuscript and all the authors made significant contributions in result presentation. HW and JZ provided funding, project administration and supervision.

Competing interests

The authors declare no competing financial interests.

Data availability statement

All the data presented in this study are available by consulting to Dr. Hui Wei (weihui@scau.edu.cn).

Electronic supplementary material

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