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Influence of nitrogen sources on the tolerance of *Lacticaseibacillus rhamnosus* to heat stress and oxidative stress

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Abstract: It has been found that 32 genes related to nitrogen source metabolism in *Lacticaseibacillus rhamnosus* are downregulated under both heat stress and oxidative stress. In this study, the influence of different nitrogen sources within the growth medium on the tolerance of *L. rhamnosus* to heat stress and oxidative stress was investigated. Tryptone-free MRS was found to enhance the tolerance of *L. rhamnosus* hsryfm 1301 to heat stress and oxidative stress during the whole growth period, and this result was universal for all *L. rhamnosus* species analyzed. The strongest strengthening effect occurred when the OD_{600} value reached 2.0, at which the survival rates under heat stress and oxidative stress increased 130-fold and 40-fold, respectively. After supplementing phenylalanine, isoleucine, glutamate, valine, histidine, or tryptophan into the tryptone-free MRS, the tolerance of *L. rhamnosus* to heat stress and oxidative stress exhibited a sharp drop. The spray drying survival rate of *L. rhamnosus* hsryfm 1301 cultured in the tryptone-free MRS rose to 75% (from 30%), and the spray dried powder also performed better in the experimentally simulated gastrointestinal digestion. These results showed that decreasing the intake of amino acids is an important mechanism for *L. rhamnosus* to tolerate heat stress and oxidative stress. When *L. rhamnosus* is cultured for spray drying, the concentration of the nitrogen source's components should be an important consideration.

Keywords: nitrogen sources, Lacticaseibacillus rhamnosus, heat stress, oxidative stress, spray drying

Introduction

Lacticaseibacillus rhamnosus is a known probiotic species. The probiotic properties of *L. rhamnosus* strains, such as the ability to adjust intestinal microbiota, lower blood lipid levels, and enhance natural and acquired immunity, have been previously described (Chen et al. 2014; Segers and Lebeer 2014; Toscano et al. 2017).

Spray drying is cost-effective and highly flexible, and it is commonly used in the food industry (Martín et al. 2015). Therefore, spray drying has been applied in the production of *L. rhamnosus* powder (Prasad et al. 2003; Moayyedi et al. 2018; Agudelo-Chaparro et al. 2021). However, spray drying results in a lower survival rate of starters compared to freeze-drying because the bacterial cells are exposed to severe heat and oxidative stress. It has been shown that the survival rate of lactic acid bacteria (LAB) in spray drying correlates with their combined robustness under heat and oxidative stress (Dijkstra et al. 2014; Simpson et al. 2005).

Lacticaseibacillus rhamnosus possesses a stress-inducible defense system against heat stress and oxidative stress (Zhang et al. 2018). Several proteins in *L. rhamnosus* HN001, including GroEL, DnaK, and several glycolytic enzymes, are regulated after heat shock; as a result, *L. rhamnosus* HN001 shows a significantly higher survival rate after pretreatment with heat stress (Prasad et al. 2003). In our previous study, it was found that either heat stress or oxidative stress enhanced the tolerance of *L. rhamnosus* hsryfm 1301 to both heat stress and oxidative stress (Zhang et al. 2018). Transcriptome-phenotype matching was implemented to reveal the mechanism of the cross-adaptation of *L. rhamnosus* hsryfm 1301 to heat stress and oxidative stress. The overlapping differently expressed genes (DEGs) were mainly classified into amino acid or oligopeptide ABC transporters, amino acid metabolism enzymes, and peptidases, and they were all downregulated (Zhang et al. 2020). However, amino acids often play a positive role in stress resistance of LAB (Fernández and Zúñiga 2006; Yang et al. 2021). Therefore, it is interesting to determine whether a decrease in the nitrogen source would improve the survival of *L. rhamnosus* hsryfm 1301 under heat stress and oxidative stress.

In the present study, the changes in the survival rate of *L. rhamnosus* hsryfm 1301, under heat stress, oxidative stress, or during spray drying were investigated by reducing the concentration of the nitrogen source to identify the influence of the nitrogen sources in the growth medium on the tolerance of *L. rhamnosus* to heat stress and oxidative stress.

Materials and methods

Bacterial strains and growth conditions

Lacticaseibacillus rhamnosus hsryfm 1301 (CGMCC No. 8545) was isolated from the gut of a centenarian from Bama, Guangxi Province, China in our previous study (Chen et al. 2014). The other *Lactobacillus* strains in this study are listed in Table 1. All the strains were cultured in MRS broth (2% (v/v) inoculation) at 37°C under static incubation.

The four types of modified MRS media (tryptone-free MRS, low-tryptone MRS, MRS, or high-tryptone MRS, Table 2) were adjusted

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Table 1. Strains used in this study.

Species	Strain	Source
L. rhamnosus	hsryfm 1301	Chinese centenarian; CGMCC no. 8545
L. rhamnosus	LV108	Chinese centenarian; CGMCC no. 1531
L. rhamnosus	GG	Commercially available probiotics Culturelle®
L. rhamnosus	HN001	Commercially available probiotics Pe-delight®
L. rhamnosus	grx10	Chinese centenarian, CGMCC no. 2526
L. rhamnosus	SP1	Commercially available starter
L. rhamnosus	LY0	Commercially available starter
L. fermentum	grx08	Chinese centenarian; CGMCC no. 7695
L. casei	Shirota	Commercially available drink Yakult®
L. paracasei	Myt	Commercially available drink 每益添® (2019)
L. plantarum	58	Chinese centenarian, wild strain

Table 2. Concentrations of tryptone peptone in the modified MRS media.

Media name	Tryptone peptone/g l^{-1}
tryptone-free MRS	0
low-tryptone MRS	0.625
MRS	10.0
high-tryptone MRS	20.0

by adding different amounts of tryptone (Zhang et al. 2020). The amino acid supplemented medium was prepared with the addition of Glu, Asp, Thr, Ser, Cys, Met, Ala, Gly, His, Arg, Lys, Pro, Val, Leu, Ile, Phe, Tyr, or Trp to the tryptone-free MRS medium (final concentration was 0.8 g l^{-1}).

The base formulation for the MRS medium is as follows: glucose 20 g l^{-1}, tryptone 10 g l^{-1}, beef extract 5.0 g l^{-1}, yeast extract 2.5 g l^{-1}, sodium acetate 2.5 g l^{-1}, ammonium citrate 1.0 g l^{-1}, K_2PO_4 1.0 g l^{-1}, $MgSO_4 \bullet 7H_2O$ 1.0 g l^{-1}, $MnSO_4 \bullet H_2O$ 0.025 g l^{-1}, Tween-80 0.30 ml l^{-1}.

Measurement of heat stress and oxidative stress tolerance

Oxidative stress (1.6 mmol l^{-1} H_2O_2, 1 h) and heat stress (54°C, 1 h) were implemented according to our previous study (Zhang et al. 2018). The colony forming unit (CFU) counts of the treated samples were calculated according to a previously described drop plate technique (Zhang et al. 2019). The colony number of every drop (N_{colony}) was then counted. When 1 g of spray dried powder was mixed with 9 ml normal saline, the total volume of the solution was 9.453 ml. The CFU count was calculated using the following equation:

$$\mathrm{CFU ml^{-1}} = 10^T \times 200 \times N_{colony},$$
$$\mathrm{CFU\, g^{-1}} = 0.9453 \times 10^T \times 200 \times N_{colony}.$$

Stress tolerance of *L. rhamnosus* hsryfm 1301 at different growth stages

After the overnight incubation, *L. rhamnosus* hsryfm 1301 was cultured in tryptone-free MRS broth, low-tryptone MRS broth, MRS broth, or high-tryptone MRS broth and incubated at 37°C. When the OD_{600} values reached 0.5 (7×10^7 CFU ml^{-1}), 1.0 (2×10^8 CFU ml^{-1}), 2.0 (3.8×10^8 CFU ml^{-1}), 3.0 (4.8×10^8 CFU ml^{-1}), and 4.0 (9.5×10^8 CFU ml^{-1}), the cultures were treated under heat stress and oxidative stress and the survival rates of the strains were measured.

Stress tolerance of *L. rhamnosus* in amino acid supplement mediums

After the overnight incubation, *L. rhamnosus* hsryfm 1301 was cultured in broth supplemented with each amino acid and incubated at 37°C until the OD_{600} values reached 2.0. The cultures were treated under heat stress and oxidative stress, and the survival rates of the strains were measured.

Stress tolerance of other strains in low nitrogen source cultures

After the overnight incubation, six *L. rhamnosus* strains and four other LAB strains were diluted 50-fold in 5 ml of tryptone-free MRS broth, low-tryptone MRS broth, MRS broth, or high-tryptone MRS broth and incubated at 37°C until the OD_{600} values reached 0.5 and 2.0. The cultures were treated under heat stress and oxidative stress, and the survival rates of the strains were measured.

The survival rate of *L. rhamnosus* during spray drying

After the overnight incubation, *L. rhamnosus* hsryfm 1301 was cultured in 600 ml of tryptone-free MRS broth and MRS broth and incubated until the OD_{600} values reached 2.0. The cultures were harvested (6000 g, 5 min, 37°C) and re-suspended in 600 ml of protective agent (skim milk powder 150 g l^{-1}, trehalose 90 g l^{-1}, glycerol 8 ml l^{-1}). Cell suspensions were spray dried in a laboratory scale spray dryer (GEA spray dryer MOBILE MINOR, Dusseldorf, Germany) by using a constant inlet air temperature of 120°C, an outlet temperature of 60°C, and a flux of 510 ml h^{-1}.

Three independent replicates were performed for each strain. Spray dried powders were vacuum sealed in individual samples of 1 g (Paéz et al. 2012). Cell counts of *L. rhamnosus* hsryfm 1301 were performed before and after spray drying on MRS agar and periodically during storage at –20, 4, or 25°C for 120 days. The survival rate was calculated using the following equation:

$$\text{Survival Rate} = \frac{\text{Total CFU of powder}}{\text{Total CFU of suspension}} = 0.24 \times \frac{\text{CFU of 1g powder}}{\text{CFU of 1ml suspension}}.$$

Resistance of spray dried powder to simulated gastrointestinal digestion

To study the resistance to simulated gastrointestinal digestions, spray dried powder (1 g) was mixed with 9 ml phosphate buffered saline (PBS), and 1 ml cell suspension was mixed with 9 ml simulated gastric fluid (consisting of 3.0 mg ml^{-1} pepsin in 0.086 mol l^{-1} NaCl at pH 2.5) or 9 ml MRS broth supplemented with 0.1% bile salt. Samples (1 ml) were removed for the cell count after mixing and 3 h of incubation at 37°C in a water bath for cell count. The survival rate was calculated using the following equation:

$$\text{Survival Rate} = \frac{\text{CFU of the sample at 3 h}}{\text{CFU of the sample at 0 h}}.$$

Statistical analysis

All stress resistance assays were repeated three times. Significant differences between the two values were evaluated by unpaired the Student's *t*-test ($P < 0.05$).

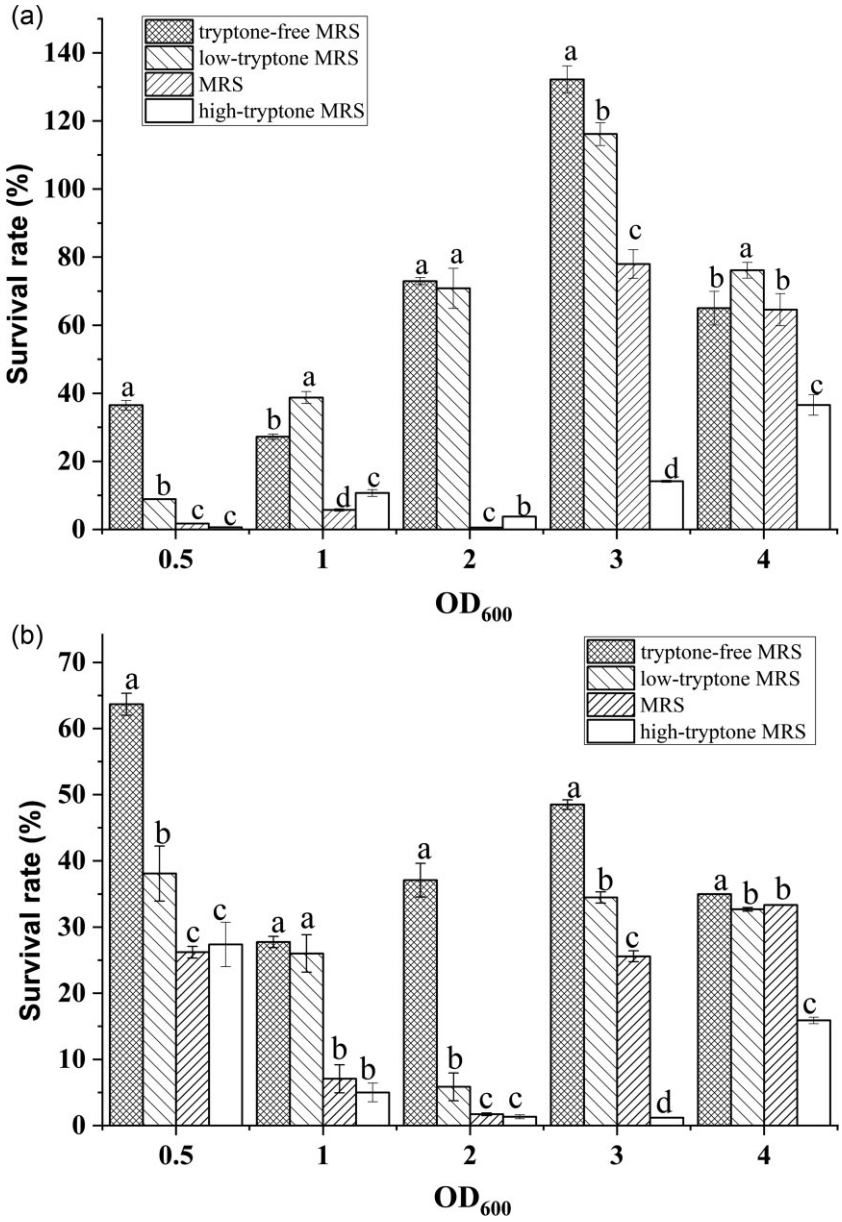

Fig. 1 Tolerance of *L. rhamnosus* to heat stress and oxidative stress in the modified MRS broths. The concentration of tryptone differed in each modified MRS broth. Cells were harvested when the OD_{600} values reached 0.5, 1.0, 2.0, 3.0, and 4.0. (**a**) Tolerance of *L. rhamnosus* hsryfm 1301 to heat stress (54°C, 1 h). (**b**) Tolerance of *L. rhamnosus* hsryfm 1301 to oxidative stress (1.6 mmol l^{-1} H_2O_2, 1 h). $^{a\text{-}d}$Values within each column with different superscripts are significantly different ($P < 0.05$).

Results

Influence of tryptone on the tolerance of *L. rhamnosus* hsryfm 1301 to heat stress and oxidative stress

Lacticaseibacillus rhamnosus hsryfm 1301 was cultured in tryptone-free MRS broth, low-tryptone MRS broth, MRS broth, or high-tryptone MRS broth. The growth characteristics of *L. rhamnosus* hsryfm 1301 were influenced by tryptone. The biomass of *L. rhamnosus* hsryfm 1301 was lower in the tryptone-free MRS broth and the low-tryptone MRS broth, but the OD_{600} values of all the cultures reached 4.0 in 12 h. *Lacticaseibacillus rhamnosus* hsryfm 1301 exhibited a similar growth rate until the OD_{600} values reached 2.0 (Zhang et al. 2020). The heat stress tolerance of *L. rhamnosus* hsryfm 1301 in the MRS broth varied significantly at different growth stages. After a heat stress treatment (54°C, 1 h), the survival rate of cells cultured in the MRS broth was <10% before the OD_{600} values reached 2.0 (exponential phase). However, the survival rates of cells from the MRS broth culture whose OD_{600} value reached 3.0 rose to 75% (Figure 1a). Interestingly, the heat stress tolerance of the samples from the tryptone-free MRS broth was much stronger than those from the high-tryptone MRS broth at the same OD_{600} value. Especially when the OD_{600} value was 2.0 (exponential phase), the tryptone-free MRS broth showed the strongest strengthening effect on the heat stress tolerance of *L. rhamnosus* hsryfm 1301 (from 2% to 75%) (Figure 1a).

The oxidative stress tolerance of *L. rhamnosus* hsryfm 1301 in the MRS broth also varied significantly at different growth stages. However, unlike the heat stress treatment, oxidative the stress treatment (1.6 mmol l^{-1} H_2O_2, 1 h) caused the most serious viable count loss when the OD_{600} values reached 2.0, at which the

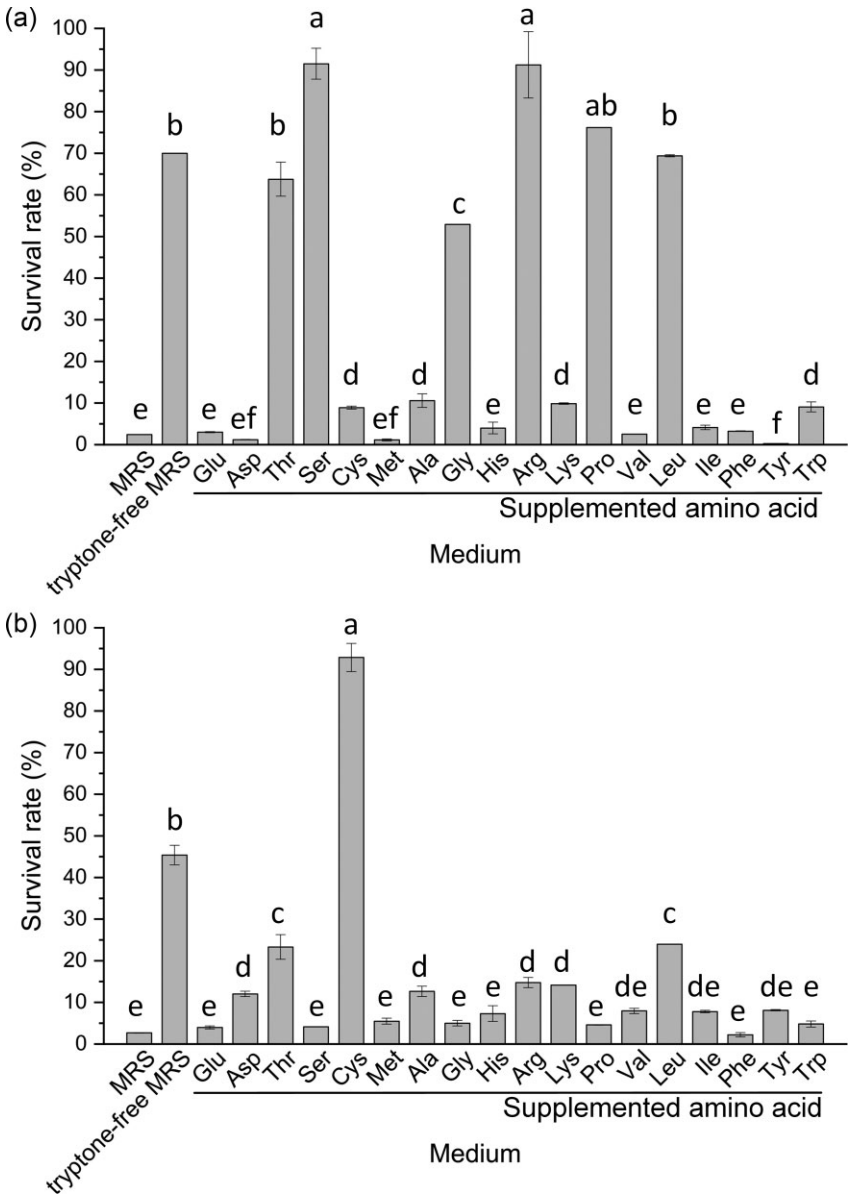

Fig. 2 Tolerance of *L. rhamnosus* to heat stress and oxidative stress in the amino acid supplemented mediums. The supplemented concentration of each amino acid was 0.8 g l^{-1}. (**a**) Tolerance of *L. rhamnosus* hsryfm 1301 to heat stress (54°C, 1 h). (**b**) Tolerance of *L. rhamnosus* hsryfm 1301 to oxidative stress (1.6 mmol l^{-1} H_2O_2, 1 h). $^{a\text{-}f}$Values within each column with different superscripts are significantly different ($P < 0.05$).

survival rates of cells were <5%. But the survival rates of cells from the MRS broth culture were >25% when the OD_{600} value was 0.5, 3.0, and 4.0 (Figure 1b). Interestingly, the heat stress and oxidative stress tolerance of all the samples from the tryptone-free MRS broth were much stronger than those from the high-tryptone MRS broth at the same OD_{600} value. Especially when the OD_{600} value was 2.0 (exponential phase), tryptone-free MRS broth showed the strongest strengthening effect on the oxidative stress tolerance of *L. rhamnosus* hsryfm 1301 (from 3% to 35%) (Figure 1b). In summary, tryptone decreased the tolerance of *L. rhamnosus* hsryfm 1301, to both heat stress and oxidative stress at different growth stages.

Key amino acids decreasing the tolerance of *L. rhamnosus* to heat stress and oxidative stress

It was unknown whether tryptone would be degraded into amino acids in the process of decreasing the tolerance of *L. rhamnosus* to heat stress and oxidative stress. Therefore, the tolerance of *L. rhamnosus* hsryfm 1301 to heat stress and oxidative stress was investigated in amino acid supplement medium broth. Thr, Ser, Gly, Pro, Arg, and Leu did not influence the thermotolerance of *L. rhamnosus* hsryfm 1301. All other amino acids significantly decreased the thermotolerance of *L. rhamnosus* hsryfm 1301. In particular, only 1% of cells cultured in Met, Asp, or Tyr supplement medium broth survived after treatment with heat stress (54°C, 1 h) (Figure 2a). On the other hand, more amino acids influence the oxidative stress tolerance of *L. rhamnosus* hsryfm 1301. Except for Cys, which increased the survival rate of *L. rhamnosus* hsryfm 1301 from 45% to 93%, all other amino acids tested decreased the oxidative stress tolerance of *L. rhamnosus* hsryfm 1301. The weakening effect on the oxidative stress tolerance of Ser and Leu was less significant (Figure 2b).

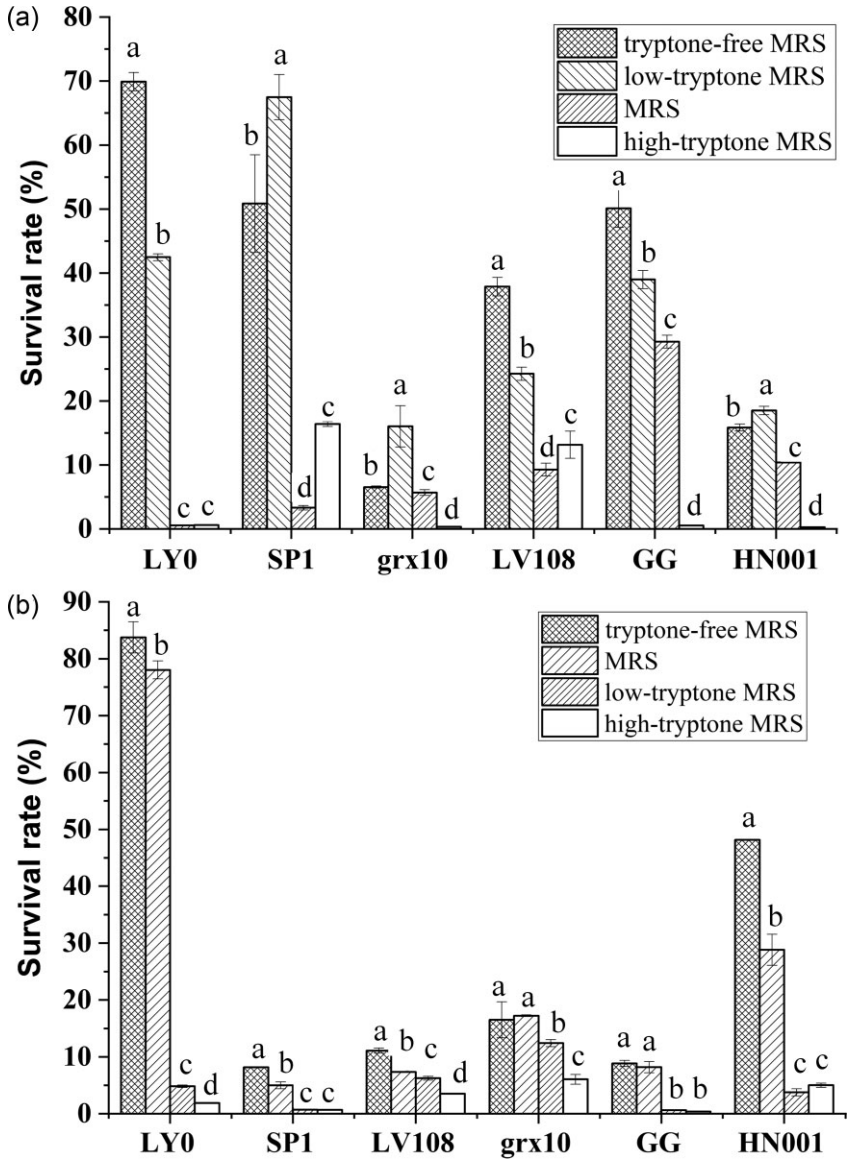

Fig. 3 Heat stress and oxidative stress tolerance of the six other *L. rhamnosus* strains in the modified MRS broths. The concentration of tryptone differed in each modified MRS broth. (**a**) Tolerance of other *L. rhamnosus* strains to heat stress (54°C, 1 h). (**b**) Tolerance of other *L. rhamnosus* strains to oxidative stress (1.6 mmol l^{-1} H_2O_2, 1 h). $^{a\text{-}d}$Values within each column with different superscripts are significantly different ($P < 0.05$).

Influence of tryptone on the heat stress and oxidative stress tolerance of other *L. rhamnosus* strains

Six other *L. rhamnosus* strains, 1 *Lacticaseibacillus casei* strain, 1 *Lacticaseibacillus paracasei* strain, 1 *Lactiplantibacillus plantarum* strain, and 1 *Limosilactobacillus fermentum* strain were cultured in tryptone-free MRS broth, low-tryptone MRS broth, MRS broth, or high-tryptone MRS broth, respectively. When the OD_{600} values reached 2.0, the tolerance to heat stress and oxidative stress of all the six *L. rhamnosus* strains decreased with increasing tryptone concentration. Only the thermotolerance of *L. rhamnosus* grx10 was weaker in the tryptone-free MRS than in the low-tryptone MRS broth (still stronger than that in high-tryptone MRS broth) (Figure 3a). Therefore, a low nitrogen source environment enhanced the heat stress and oxidative stress tolerance of all tested *L. rhamnosus* strains. Among them, the heat stress tolerance of *L. rhamnosus* LY0, SP1, and GG was influenced more significantly, and the oxidative stress tolerance of *L. rhamnosus* LY0 and *L. rhamnosus* HN001 was influenced more significantly (Figure 3b).

Lacticaseibacillus paracasei Myt performed exhibited a similar thermotolerance in four kinds of mediums, as did *L. casei* Shirota. Interestingly, *L. paracasei* Myt exhibited a stronger thermotolerance when the OD_{600} value was 0.5 (Figure 4a), while *L. casei* Shirota exhibited a stronger heat stress tolerance when the OD_{600} value was 2.0 (Figure 4b). The thermotolerance of *L. plantarum* 58 and *L. fermentum* grx08 was stronger in the low-tryptone MRS broth than in the other three mediums only when the OD_{600} values reached 2.0 (Figure 4c and d). On the other hand, no significant correlation between the oxidative stress tolerance of *L. casei* Shirota, *L. paracasei* Myt, *L. plantarum* 58, or *L. fermentum* grx08 and the concentration of tryptone was found regardless of whether the OD_{600} value was 0.5 or 2.0 (Figure 5). Only *L. paracasei* Myt exhibited a slightly stronger oxidative stress tolerance in a low nitrogen source environment when the OD_{600} value was 2.0. The strengthening effect on heat stress and oxidative stress tolerance

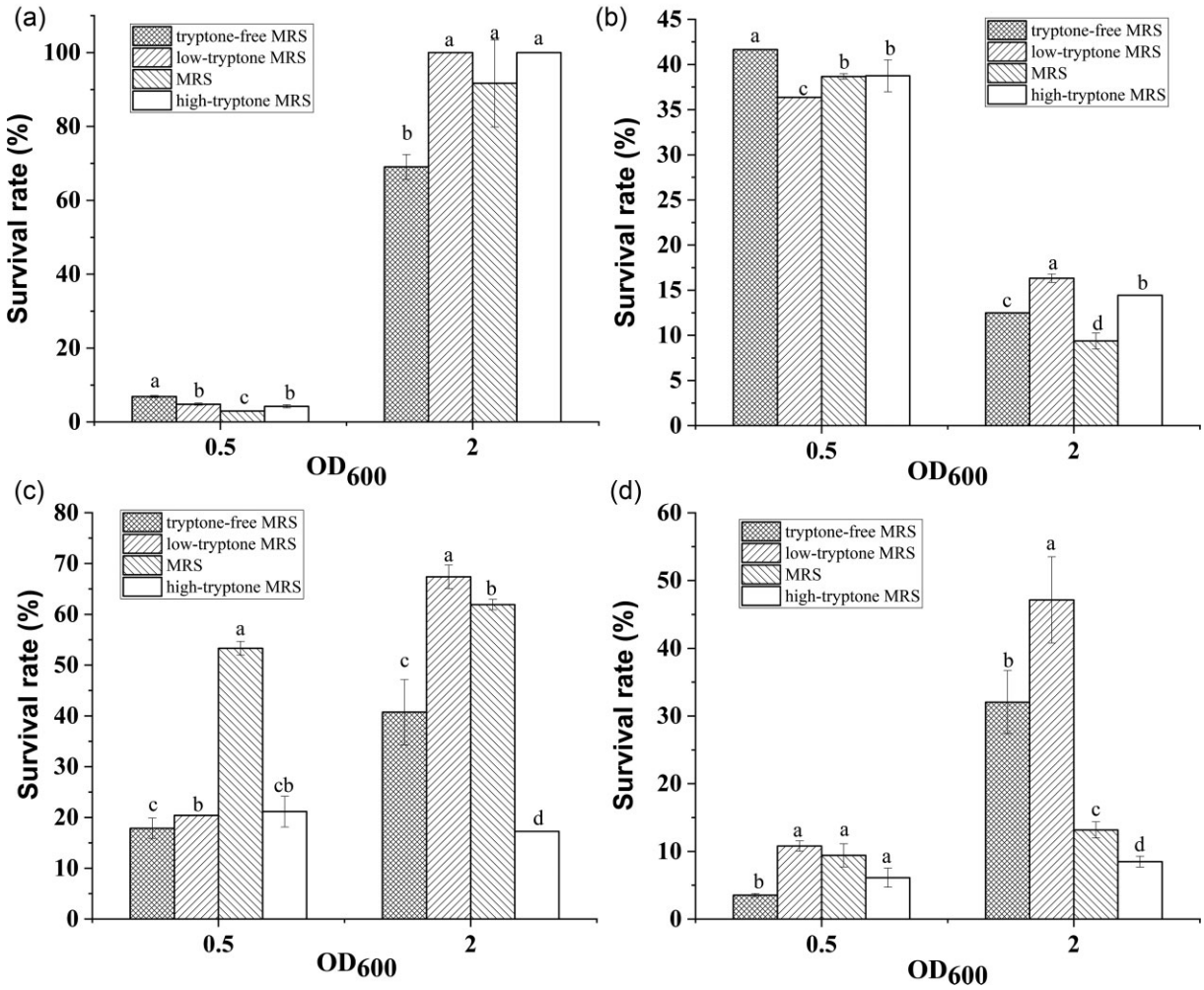

Fig. 4 Heat stress tolerance of other *Lactobacillus* species in the modified MRS broths. The concentration of tryptone differed in each modified MRS broth. Cells were harvested when the OD_{600} values reached 0.5 and 2.0. The heat stress performed was 54°C, 1 h. (**a**) *L. casei* Shirota. (**b**) *L. paracasei* Myt. (**c**) *L. plantarum* 58. (**d**) *L. fermentum* grx08. [a-d]Values within each column with different superscripts are significantly different ($P < 0.05$).

by a low nitrogen source environment was not widespread among the *Lactobacillus* species.

Influence of tryptone on the spray dried powder of *L. rhamnosus* hsryfm 1301

For cells were always in chains, the CFU of *L. rhamnosus* hsryfm 1301 cultured at OD_{600} 2.0 only reached 10^8 ml^{-1}. After spray drying, the survival rate of *L. rhamnosus* hsryfm 1301 cultured in the MRS broth was ~29.6%, while the survival rate of *L. rhamnosus* hsryfm 1301 cultured in the tryptone-free MRS broth reached 74.7% (Figure 6a). Therefore, the low nitrogen source cultures helped *L. rhamnosus* tolerate heat and oxidative stress during spray drying. The *L. rhamnosus* hsryfm 1301 cells harvested from the MRS broth tolerated acid stress well (Zhang et al. 2018), but only 0.8% of cells of *L. rhamnosus* hsryfm 1301 in the spray dried powder (cultured in MRS broth) were able to survive in simulated gastric fluid. Interestingly, the spray dried powder of *L. rhamnosus* hsryfm 1301 produced with cells harvested from the tryptone-free MRS broth had a much better simulated gastric fluid tolerance (survival rate 18%) (Figure 6b). In addition, the spray dried powder of *L. rhamnosus* hsryfm 1301 produced with cells harvested from the tryptone-free MRS broth also performed better bile salt (0.1%) tolerance than that harvested from the MRS broth.

Viability of spray dried *L. rhamnosus* hsryfm 1301 during storage

The survival rate of the *L. rhamnosus* hsryfm 1301 powders obtained from both the MRS broth and the tryptone-free MRS broth was studied during 120 days of storage at –20°C, 4°C, and 25°C. A gradual diminution in cell counts was observed in the first 2 weeks for the powder from both cultures. The diminution of the *L. rhamnosus* hsryfm 1301 powders obtained from the tryptone-free MRS broth was more significant as the temperature increased. By 120 days, the highest cell counts were observed in the powders kept at –20°C (Figure 7). The low nitrogen source cultures significantly enhanced the survival of *L. rhamnosus* hsryfm 1301 during storage at –20°C (Figure 7a). For the *L. rhamnosus* hsryfm 1301 powders obtained from MRS, no significant differences in survival during storage were observed when stored at –20°C and 4°C (Figure 7a and b). No cell counts were observed for the powders obtained from both the MRS broth and the tryptone-free MRS broth by Day 30 of storage at 25°C (Figure 7c).

Discussion

Spray drying is highly reproducible and has the advantage of rapidity and a relatively low cost in the production of probiotic

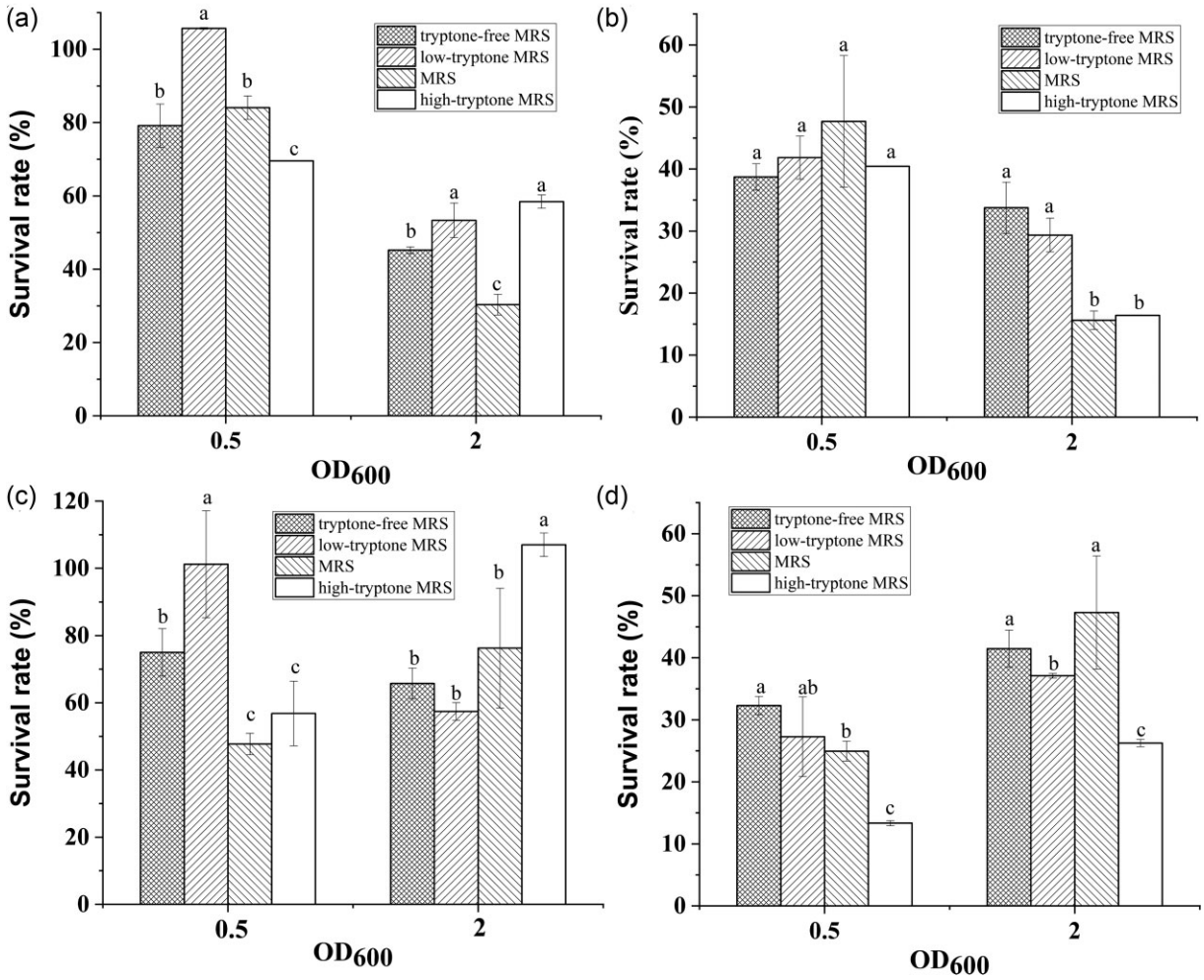

Fig. 5 Oxidative stress tolerance of other *Lactobacillus* species in the modified MRS broths. The concentration of tryptone differed in each modified MRS broth. Cells were harvested when the OD_{600} values reached 0.5 and 2.0. The heat stress performed was 1.6 mmol l^{-1} H_2O_2, 1 h. (**a**) *L. casei* Shirota. (**b**) *L. paracasei* Myt. (**c**) *L. plantarum* 58. (**d**) *L. fermentum* grx08. [a-d]Values within each column with different superscripts are significantly different ($P < 0.05$).

powder (Paéz et al. 2012; Martín et al. 2015). However, the use of high-temperature air produces severe heat and oxidative stress. The cell counts in spray dried probiotic powder are correlated with the robustness under heat and oxidative stress (Dijkstra et al. 2014).

In our previous study, it was found that up to 32 genes in *L. rhamnosus* hsryfm 1301 related to amino acid or oligopeptide ABC transport, amino acid metabolism, and peptide degradation were downregulated under both heat stress and oxidative stress (Zhang et al. 2020). In the present study, the tolerance of *L. rhamnosus* hsryfm 1301 to heat stress and oxidative stress cultured in the MRS broth and the low-tryptone MRS broth was investigated. The influence of the nitrogen sources in the growth medium on the tolerance of *L. rhamnosus* to heat stress and oxidative stress was identified in spray drying. The duration of the enhanced spray drying resistance and survival during simulated gastrointestinal digestion were also studied.

Sometimes, genes would be downregulated for energy shortage in stress-shocked cells (Papadimitriou et al. 2016). To identify whether the downregulation of genes related to nitrogen metabolism was to reduce intracellular concentration of amino acids or just to save resources and energy, the tolerance of *L. rhamnosus* to heat stress and oxidative stress cultured in different concentrations of tryptone and different growth stages was investigated. With the growth of *L. rhamnosus* hsryfm 1301, its thermotolerance gradually became much stronger, while *L. rhamnosus* hsryfm 1301 exhibited a much weaker oxidative stress tolerance only in the exponential phase. Interestingly, tryptone decreased the tolerance of *L. rhamnosus* hsryfm 1301 to heat stress and oxidative stress at each growth stage, and the strongest weakening effect for both heat stress and oxidative stress tolerance occurred when the OD_{600} value reached ~2.0 (exponential phase). These results showed that tryptone (peptides) is an important and uninvestigated factor affecting the tolerance of *L. rhamnosus* hsryfm 1301 to heat stress and oxidative stress.

It was further identified that 12 kinds of exogenous amino acids decreased the thermotolerance and 17 kinds of exogenous amino acids decreased the oxidative stress tolerance of *L. rhamnosus* hsryfm 1301, indicating that tryptone would be degraded to amino acids in the process, decreasing the tolerance of *L. rhamnosus* to heat stress and oxidative stress. Thus, when encountering heat stress or oxidative stress, *L. rhamnosus* downregulated the genes related to amino acid or oligopeptide ABC transporters, amino acid metabolism, and peptidases to decrease the intracellular amino acid concentration, so it acquired stronger heat stress and oxidative stress tolerance. This is hypothesized to be the reason for the previously observed cross-adaptation of *L. rhamnosus* hsryfm 1301 to heat stress and oxidative stress (Zhang et al. 2020).

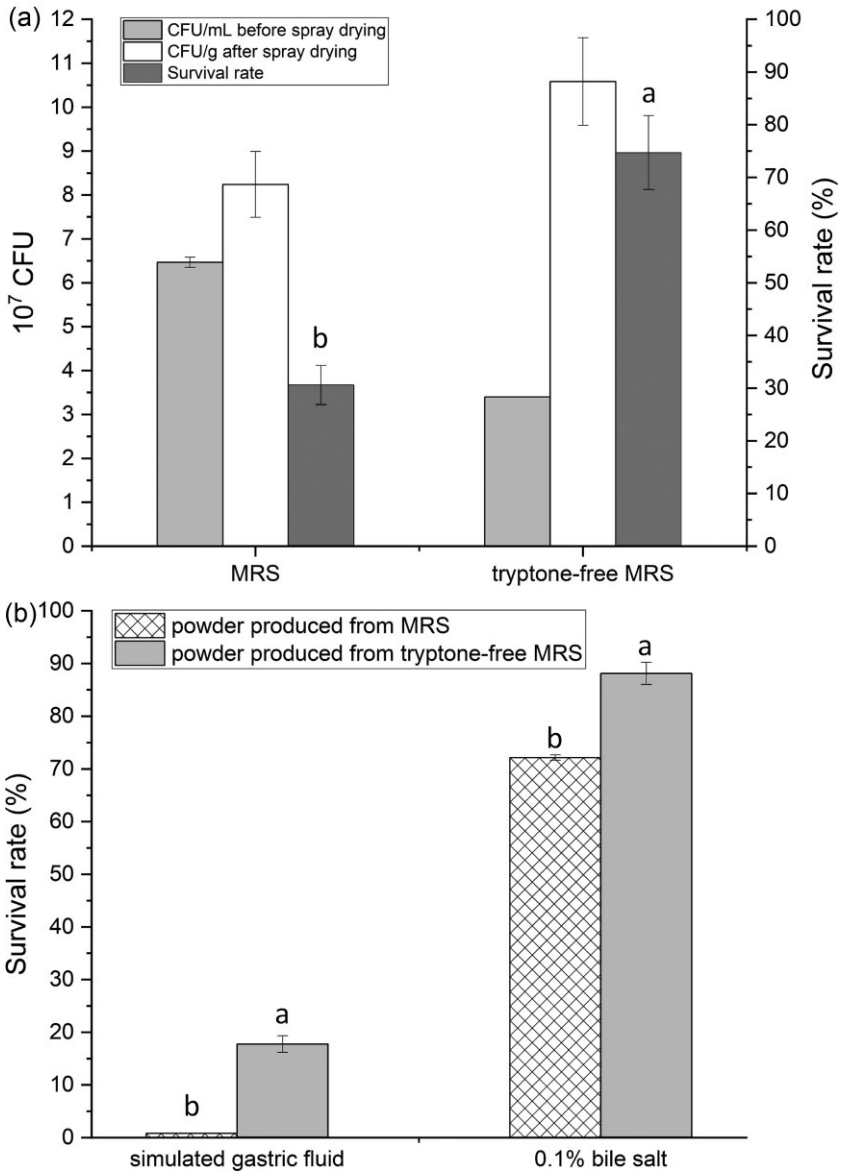

Fig. 6 Survival rate and simulated gastrointestinal resistance of the spray dried powder of *L. rhamnosus* hsryfm 1301. (**a**) Survival rate of *L. rhamnosus* hsryfm 1301 from the tryptone-free MRS broth or the MRS broth to spray drying. (**b**) Resistance of the spray dried powder of *L. rhamnosus* hsryfm 1301 from the tryptone-free MRS broth or MRS broth to the simulated gastric fluid (pH 2.5, 3 h) and bile salt (0.1%, 3 h). $^{a\text{-}b}$Values within each column with different superscripts are significantly different ($P < 0.05$).

Supplementation of different amino acids imposed distinct or sometimes opposite effects on heat or oxidative stress tolerance, which might be because there were many different pathways between the tolerance to heat stress and oxidative stress (Zhang et al. 2020). Moreover, amino acids can not only play a role in transcriptional regulation but also in protein synthesis and chemical reaction. The most obvious example was that Cys weakened the heat stress tolerance but strengthened the oxidative stress tolerance of *L. rhamnosus* hsryfm 1301, significantly due to its reducibility.

It was extraordinary that amino acids decreased the stress tolerance of *L. rhamnosus* hsryfm 1301, because amino acids often played a positive role in the stress resistance of LAB (Fernández and Zúñiga 2006; Yang et al. 2021). The conversion of Glu to GABA contributed to acid resistance in *Limosilactobacillus reuteri* (Su et al. 2011). Exogenous addition of Asp, Arg, and His significantly increased the viability of *L. casei* under acidic conditions (Broadbent et al. 2010; Wu et al. 2012). The genes in a branched-chain amino acid transport operon were upregulated by low pH in *Bifdobacterium longum* JDM301AR (Wei et al. 2019). When grown with high osmotic pressure, *Lactobacillus acidophilus* cells accumulated Pro (Jewell and Kashket 1991). Cys is a buried catalytic site in most redox enzymes (Li et al. 2019), and *L. reuteri* requires more Cys to grow optimally under aerobic conditions (Lo et al. 2009). Cys uptake and biosynthesis play important roles in resistance to the oxidative environment in *L. fermentum* and *L. acidophilus* (Calderini et al. 2017; Hung et al. 2003). In *Ligilactobacillus salivarius*, more amino acids are needed for the synthesis and repair of proteins damaged by bile (Wang et al. 2020a). The phenomenon that amino acids decreased the stress tolerance of *L. rhamnosus* might be based on the stringent response, which was described as an adaptation to amino acid starvation, heat stress tolerance, oxidative stress tolerance, and osmotic stress tolerance (Yan et al. 2009; Starosta et al. 2014). As further evidence, *L. rhamnosus* has

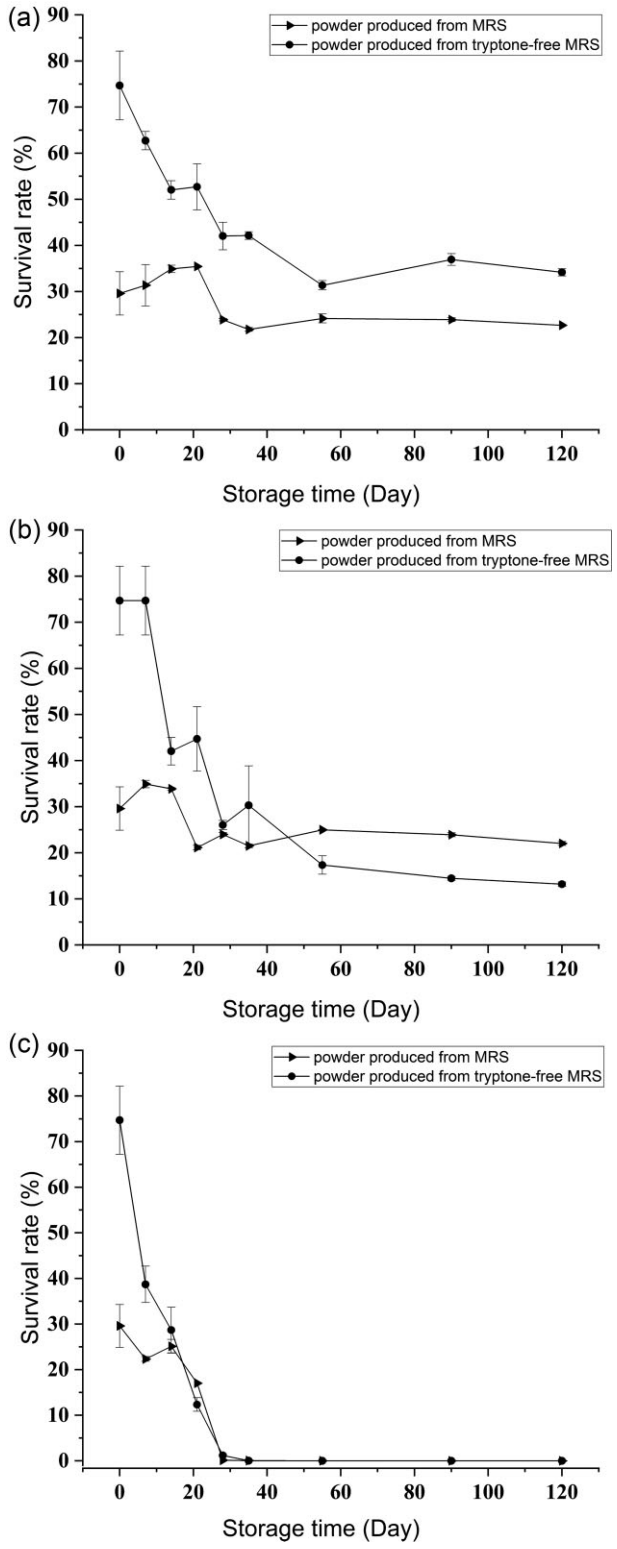

Fig. 7 Survival rate of spray dried *L. rhamnosus* hsryfm 1301 obtained from the tryptone-free MRS broth (●) or the MRS broth (▶) during the storage at –20°C (**a**), 4°C (**b**), or 25°C (**c**).

different requirements for amino acids (Sun et al. 2019), and except for branched-chain amino acids, the weakening effect on the thermotolerance was positively related to the degree of necessity of amino acids in *L. rhamnosus*. The weakening effect on the oxidative stress tolerance was not associated with the degree of necessity of amino acids, which was perhaps due to the different reducibility of amino acids; for example, Cys significantly strengthened the oxidative stress tolerance of *L. rhamnosus* hsryfm 1301.

To our knowledge, the weakening effect of nitrogen sources in the growth medium on heat stress and oxidative stress tolerance has not been reported in other LABs. Interestingly, the heat stress and oxidative stress tolerance of six different *L. rhamnosus* strains isolated from different countries (Zhang et al. 2021) decreased with increasing tryptone content. Therefore, this phenomenon is most likely universal for *L. rhamnosus*. On the other hand, although *L. casei* and *L. paracasei* are closely related to *L. rhamnosus* (collectively referred to as the *Lacticaseibacillus* group) (Huang and Huang 2018), their heat stress and oxidative stress tolerance were not strengthened in a low nitrogen source environment, suggesting that the weakening effect of nitrogen sources in the growth medium on heat stress and oxidative stress tolerance was not common in *Lactobacillus*. The stress tolerance of *Lactobacillus* showed remarkable species-specificity, which would be based on to reveal potential novel genes by comparing their genomes and transcriptomes.

Spray drying resulted in multiple stresses, mainly including heat stress and oxidative stress, which were stronger than the conditions used in this study. Therefore, whether the strengthened heat stress and oxidative stress tolerance through low nitrogen culture can help *L. rhamnosus* hsryfm 1301 survive in spray drying needs to be verified. As a result, a low nitrogen environment was effective in reducing the cell death associated with spray drying and gastrointestinal digestion. The viable count diminution of the *L. rhamnosus* hsryfm 1301 powders obtained from the tryptone-free MRS broth was more significant during storage as the temperature increased. This might be resulted from that metabolism can continue at a high temperature, which destroys the homeostasis of cells in powder where water is unavailable. Therefore, spray dried powder should be stored at a lower temperature. Nitrogen sources are essential to the high cell density culture of *L. rhamnosus* (Wang et al. 2020b). Therefore, when *L. rhamnosus* cells are prepared to produce spray dried powder, the concentration of the nitrogen source components should be optimized to achieve the right balance between biomass and survival. The mechanism of weakening heat stress and oxidative stress tolerance should be discovered so that the adverse effect on spray drying resulting from the nitrogen source can be relieved.

In summary, a low nitrogen source environment enhanced the tolerance of *L. rhamnosus* to heat stress and oxidative stress during the whole growth period, and most amino acids resulted in a sharp drop in the tolerance of *L. rhamnosus* hsryfm 1301 to heat stress and oxidative stress, suggesting that the heat stress and oxidative stress tolerance of *L. rhamnosus* can be enhanced by decreasing the intake of amino acids. The application of a low nitrogen source medium to enhance survival during spray drying and storage of powders could potentially be used at the industrial level. Before the mechanism of nitrogen source weakening heat stress and oxidative stress tolerance was discovered, the concentration of nitrogen source components should be optimized to achieve the right balance between the biomass and survival of *L. rhamnosus*.

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Conflict of interest

The authors declare no conflict of interests.

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