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RESEARCH ARTICLE

Effect of Mn^{2+} and Fe^{2+} on the Aerobic Growth of a *Lactobacillus delbrueckii* Strain Isolated from Buffalo Curd

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ABSTRACT

Lactic acid bacteria (LAB) exhibit limited tolerance to oxygen, and metal ions such as Mn^{2+} and Fe^{2+} have been suggested to play roles in oxidative stress responses. This study investigated whether supplementation with Mn^{2+} or Fe^{2+} enhances the aerobic growth of a Lactobacillus delbrueckii strain isolated from traditionally fermented buffalo milk (curd) in Sri Lanka. The isolate was pre-treated with Mn^{2+} or Fe^{2+} under anaerobic conditions and subsequently grown under aerobic conditions. Growth was assessed by measuring acid production (pH reduction) and colony-forming units (CFUs). No statistically significant differences ($p>0.05$) were observed in either acid production or CFU counts between metal-treated and untreated cultures. These results indicate that supplementation with Mn^{2+} or Fe^{2+} did not confer a measurable growth advantage under the conditions tested. However, as antioxidant mechanisms were not directly assessed, further studies incorporating enzymatic assays and varying metal concentrations are required to clarify the role of these ions in oxidative stress tolerance in LAB.

***Keywords**: Antioxidant activity of Mn^{2+}, Curdled buffalo milk, Lactobacillus delbrueckii, Mn^{2+}-catalase, Superoxide dismutase*

INTRODUCTION

Lactic acid bacteria (LAB) are a Gram-positive, non-spore-forming group of bacteria belonging to the order Lactobacillales, characterized by the production of lactic acid as the major end product of fermentation. Although some LAB exhibit limited respiratory activity under specific conditions, where components of an electron transport chain are available (Brooijmans *et al.,* 2009; Lechardeur *et al.,* 2011; Zotta *et al.,* 2017), energy generation in these organisms predominantly occurs via fermentation.

Lactic acid bacteria exhibit varying degrees of oxygen tolerance, and many strains have reduced growth under normal atmospheric oxygen concentration. Their limited ability to cope with oxygen is associated with comparatively less efficient antioxidant mechanisms to counteract reactive oxygen species (ROS). Nevertheless, some LAB possess antioxidant systems that enable survival under low-oxygen conditions.

Among these mechanisms, certain LAB species produce superoxide dismutase (SOD), which neutralizes superoxide radicals generated upon exposure to oxygen (Hosono *et al.,* 1991; Zotta *et al.,* 2017; Serata *et al.,* 2019; Wang and Li, 2022). In some cases, SOD activity is manganese-dependent and requires the presence of Mn^{2+} ions in the growth medium (Archibald and Fridovich, 1981; Gonzalez *et al.,* 1989). Additionally, some LAB strains that lack SOD can accumulate Mn^{2+} intracellularly, allowing non-enzymatic scavenging of reactive oxygen species (Archibald and Fridovich, 1981). This Mn-dependent oxidative stress resistance has been widely recognized (Kehres and Maguire, 2003; Zotta *et al.,* 2017).

Other antioxidant systems have also been reported, including Mn^{2+}dependent catalase activity (Peacock and Hassan, 2021) and NADH peroxidase systems (Watanabe *et al.,* 2016), both of which contribute to the detoxification of hydrogen peroxide. If not efficiently detoxified, hydrogen peroxide can undergo metal-catalyzed reactions, resulting in highly reactive hydroxyl radicals. Furthermore, different SOD enzymes may utilize various metal cofactors and may be differentially expressed depending on environmental conditions (Frye *et al.,* 2022).

Lactic acid bacteria are widely used in the food industry, particularly in milk fermentation processes. Curd is produced by the fermentation of milk from *Bubalus bubalis* (Water buffalo), in which casein is the predominant protein. During fermentation, LAB convert lactose into organic acids, leading to coagulation of casein and formation of a semisolid product. This process is widely practiced as a cottage industry in many parts of Asia, including Sri Lanka.

In traditional curd preparation, the starter culture is not a pure culture but a consortium of LAB derived from previously prepared curd. Fermentation typically occurs in open containers without controlled anaerobic conditions, resulting in largely aerobic environments, although deeper layers may remain relatively anaerobic (Holzapfel, 2002; Tamime and Robinson, 2007). Lactic acid bacteria differ in their ability to tolerate oxidative stress, and this variability may be influenced by the availability of metal ions such as Mn^{2+} or Fe^{2+}. However, their effects are not consistent across strains.

Therefore, the objective of this study was to isolate and identify a lactic acid bacterium from traditionally prepared curd and to assess whether supplementation with Mn^{2+} or Fe^{2+} improves its growth under aerobic conditions. These considerations may have implications for traditional curd production, although the role of metal ion supplementation still remains to be experimentally validated.

MATERIALS AND METHODS

Sample collection

A commercially available brand of buffalo curd, produced the previous night in Southern Sri Lanka, was purchased from a local retail outlet in a Colombo suburb and transported to the laboratory under ambient conditions. The sample was processed within four hours of purchase for the isolation of LAB. The study was conducted using this curd sample, from which a representative LAB isolate was selected for further analysis.

Isolation of LAB

Lactic acid bacteria were isolated from the curd sample by serial dilution (10^{-1} to 10^{-5}) followed by plating on De Man, Rogosa and Sharpe (MRS) agar (Himedia, India), as described by De Man *et al.* (1960). Serial dilutions and plating were carried out in replicate, and the plates were incubated at 37°C for 48 h under anaerobic conditions.

Identification of LAB

After incubation on MRS agar at 37°C for 48 h under anaerobic conditions, colonies with typical *Lactobacillus* morphology were observed. The selected colony was small, circular, smooth, convex, and creamy-white in appearance. A representative colony was selected and purified.

The isolate was identified based on morphological and biochemical characteristics, including Gram staining (Gram-positive rods), catalase test (negative), and carbohydrate fermentation profile consistent with *Lactobacillus* spp. The identification was further confirmed by 16S rRNA gene sequencing. Genomic DNA was extracted from the purified bacterial isolate (Wizard Genomic DNA Purification Kit, Promega, USA). The 16S rRNA gene was amplified using the universal primers 27F and 1492R, which are specific to the rRNA gene in bacteria. The PCR product was purified and sequenced commercially. The resulting sequence was analysed using the BLAST algorithm against the NCBI GenBank database.

Growth of the isolate in the presence of metal ions (pre-treatment with metal ions)

The isolate was grown in MRS broth supplemented separately with Fe^{2+} (added as $FeSO_4.7H_2O$, 1.0 gL^{-1}) and Mn^{2+} (added as $MnSO_4.7H_2O$, 0.5 gL^{-1}) at 37°C for 12 h under anaerobic conditions. The reported concentrations refer to the added metal salts. MRS medium inherently contains Mn^{2+} (approximately 0.05 g L^{-1} as $MnSO_4$ in the original formulation); therefore, Mn^{2+} supplementation increased the total manganese concentration in the medium.

Equal volumes of inoculum (1.0 mL of overnight culture) were used to ensure comparable initial cell densities. The isolate grown in MRS broth under identical conditions without additional metal supplementation was used as the control.

The use of Fe^{2+}or Mn^{2+} supplementation is consistent with previous studies investigating the role of metal ions in oxidative stress responses and metabolism in LAB (Archibald and Fridovich, 1981; Gonzalez *et al.,* 1989; Frye *et al.,* 2022).

Determination of acid production under aerobic conditions by the isolate pre-treated with metal ions

Aliquots (1.0 mL) from 12 h pre-treated cultures were transferred separately into 10 mL of sterile MRS broth and incubated at 37°C for 12 h under shaking (10 rpm) aerobic conditions. The anaerobic pre-treatment step was used to standardize the physiological state of the cultures and to allow uptake of metal ions prior to assessing their response under aerobic conditions.

Lactic acid production during incubation was estimated by measuring the pH after 12 h, which served as an indirect indicator of metabolic activity and growth. The acid production under aerobic conditions by the isolate pre-treated with each metal ion was compared with that of the untreated control grown under identical conditions.

Determination of aerobic growth of the isolate pre-treated with metal ions

Aliquots (1.0 mL) from 12 h pre-treated cultures were transferred separately to sterile Petri dishes, and the MRS agar medium was poured, mixed, and incubated under aerobic conditions (pour plate method). The growth was determined in terms of colony forming units (CFUs) enumerated at 48 h. The number of CFUs produced under aerobic conditions by the isolate, which was pre-treated separately for 12 h with supplementation of each metal ion, was compared with the CFUs produced by the isolate grown under the same conditions without any pre-treatment.

Statistical analysis

The pH measurements were conducted in triplicate, while CFU enumerations were performed using six replicates. The results are presented as mean ± standard error (SE). Statistical significance between groups was evaluated using pairwise comparisons with two-sample t-tests. A p-value of <0.05 was considered statistically significant. Statistical analyses were carried out using Minitab statistical software (Version 17 for Windows).

RESULTS AND DISCUSSION

Identification of the LAB isolate

The LAB isolate was identified as *Lactobacillus delbrueckii* based on morphological and biochemical characteristics. The identification was further confirmed by 16S rRNA gene sequence analysis (performed by a commercial sequencing service), which had the highest similarity to *L. delbrueckii* reference sequences in the GenBank database.

Acid production under aerobic conditions by the isolated LAB pre-treated with metal ions

The effect of Fe^{2+} pre-treatment on acid production during subsequent aerobic growth is shown in Table 1. The mean pH reduction over 12 h in the untreated control was 1.33±0.66 (n=3), whereas in cultures pre-treated with Fe^{2+}ions, the pH reduction was 0.79±0.012 (n=3). Statistical analysis using Welch's t-test indicated no significant difference between the two conditions (p=0.49), suggesting that pre-treatment with Fe^{2+} did not enhance acid production under the conditions tested.

Table 1: The reduction of of pH during 12 h of aerobic growth of *Lactobacillus delbrueckii* following pre-treatment with metal ions

Pre-treatment condition	Time (h)	Average pH ± SE (n=3)	pH drop in 12 h ± SD
No pre-treatment	0	5.28±0.012	1.33±0.660
	12	3.95±0.657	
Pre-treated with Fe^{2+} ions	0	5.28±0.003	0.79±0.012
	12	4.49±0.012	
Pre-treated with Mn^{2+} ions	0	5.28±0.012	0.86±0.016
	12	4.42±0.012	

The effect of Mn^{2+} pre-treatment on acid production is also presented in Table 1. The mean pH reduction over 12 h in Mn^{2+}-treated cultures was 0.86±0.016 (n=3), compared to 1.33±0.66 (n=3) in the untreated control. Statistical analysis had no significant difference between the two conditions (p=0.54).

Overall, the absence of significant differences in pH reduction between treated and untreated cultures indicates that neither Mn^{2+} nor Fe^{2+} pre-treatment enhanced aerobic growth of the isolate, as assessed by acid production. However, as pH reduction is an indirect indicator of metabolic activity, these results do not provide direct evidence regarding the presence or absence of antioxidant mechanisms.

Aerobic growth of the isolate pre-treated with metal ions

The number of CFUs produced on MRS agar by 1 mL of *L. delbrueckii* culture after 12 h of growth under aerobic conditions is presented in Table 2. The increase in CFUs after 12 h was 234.17±1.97 (n=6) in the untreated control and 234.33±1.75 (n=6) in cultures pre-treated with Fe^{2+} ions. A two-sample t-test indicated no statistically significant difference between the two conditions (t=1.16, p=0.28).

Table 2: Colony-forming units (CFUs) produced by 1 mL of *Lactobacillus delbrueckii* culture after 12 h of aerobic growth following pre-treatment with metal ions

Pre-treatment condition	Time (h)	Average CFUs ± SE (n = 6)	Increase in CFUs after 12 h ± SD
No pre-treatment	0	40.83±1.301	234.17±1.97
	12	275±1.506	
Pre-treated with Fe^{2+} ions	0	40.17±1.424	234.33±1.75
	12	274.5±0.957	
Pre-treated with Mn^{2+} ions	0	40.83±0.723	233.00±3.36
	12	273.83±1.167	

The pre-treatment with ions did not help the bacterium to generate a significantly higher number of CFUs under aerobic conditions

Similarly, Mn^{2+} pre-treatment did not affect bacterial proliferation. The increase in CFUs was 233.00±3.36 (n=6), which was not significantly different from the control (p=0.64).

These findings indicate that Mn^{2+} and Fe^{2+} supplementation did not result in a measurable enhancement of aerobic growth under the conditions tested. The comparable CFU counts also suggest that the metal ions did not exert inhibitory effects at the concentrations used.

The absence of a detectable enhancement in aerobic growth may be explained by several biological factors. Oxidative stress tolerance in lactic acid bacteria is strain-dependent (Zotta *et al.,* 2017; Serata *et al.,* 2019), and the isolate may not rely significantly on metal-dependent antioxidant systems. Additionally, intracellular uptake of metal ions may be regulated, and increased extracellular concentrations may not lead to increased intracellular availability (Kehres and Maguire, 2003; Aguirre and Culotta, 2012). The presence of Mn^{2+} in the basal MRS medium may have already satisfied the metal requirements of the organism. It is also possible that the concentrations used were not optimal for enhancing antioxidant function, as the effects of manganese supplementation have been shown to vary among species and growth conditions (Archibald and Fridovich, 1981; Zotta *et al.,* 2017).

Furthermore, the isolate may utilize alternative antioxidant mechanisms, such as NADH peroxidase activity, which contribute to oxidative stress tolerance independently of Mn^{2+}- or Fe^{2+}-dependent pathways (Watanabe *et al.,* 2016; Zotta *et al.,* 2017).Therefore, the lack of response observed in this study likely reflects a combination of strain-specific physiology and experimental conditions.

It should be noted that CFU counts and pH measurements are indirect indicators and do not directly assess oxidative stress tolerance or antioxidant capacity. Therefore, while no growth enhancement was observed, the involvement of metal-dependent antioxidant mechanisms cannot be conclusively determined from the present data. Direct biochemical measurements of oxidative stress markers and antioxidant enzyme activities would be required to establish such mechanisms (Serata *et al.,* 2019; Peacock and Hassan, 2021).

CONCLUSION

It should be noted that the present study was based on a single commercially available curd sample; therefore, the findings reflect the characteristics of a specific isolate rather than the broader microbial diversity of curd products. Future studies involving multiple samples and brands would be required to assess variability and improve generalizability. Under the experimental conditions of this study, pre-treatment of the *L. delbrueckii* isolate with Mn^{2+} or Fe^{2+} did not result in a measurable growth advantage during subsequent aerobic incubation, as indicated by the absence of significant differences in both acid production and CFU counts between metal-treated and untreated cultures. These findings suggest that supplementation with Fe^{2+} or Mn^{2+}, at the concentrations tested, did not enhance the aerobic growth of the isolate. However, this should not be interpreted as evidence for the absence of metal-dependent antioxidant mechanisms, as no direct biochemical assays were performed. The results are best understood as reflecting the response of a specific strain under defined experimental conditions. Further studies incorporating a range of metal ion concentrations and direct measurements of oxidative stress and enzyme activity are required to clarify the role of metal ions in the aerobic physiology of lactic acid bacteria.

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