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Leuconostoc mesenteroides M13: optimization of bioprocesses and antioxidant property for food industry application

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Abstract

Several reactive oxygen species are related to physiological functions but also play roles in the development of certain human diseases. Reactive oxygen species are counterbalanced by a physiological defense network such as antioxidants. Probiotic strains have been used as natural antioxidants, but its viable scalability is a challenger. The objective of this work was to optimize the bioprocesses of *Leuconostoc mesenteroides* M13 and evaluate its antioxidant activity and its shelf-life. The optimized production of M13 biomass was 2,542% greater than non-optimized processes, resulting in a yield of 2.67×10^{10} CFU mL^{-1} under conditions comprising 13.67 g L^{-1} of yeast extract and .73 g L^{-1} of magnesium sulfate. The aggregated spherical structure observed in scanning electron microscopy may have a certain protective effect. The viability of the lyophilized strain storage at 4°C was maintained for a period of up to 8 months. Antioxidant activity assays revealed efficacies for intact and lysed cells as 81 and 65%, respectively. The survival rate of the M13 strain in the presence of hydrogen peroxide was observed at 86% (1.0 mM for 8 h). M13 demonstrated a cost-effective strategy for probiotic application in the food industry as an antioxidant supplement.

Keywords: antioxidant; bioprocess; probiotics; shelf-life.

Practical Application: Probiotics with antioxidant activity and stability for application in the food.

1 INTRODUCTION

The term reactive oxygen species (ROS) mainly refers to free radicals derived from molecular oxygen and a few other chemically reactive molecules, which originate during the gradual reduction of molecular oxygen (Hoffmann et al., 2021; Tang et al., 2022), causing oxidative stress (OS) (Houldsworth, 2023). OS appears if these mechanisms work incorrectly or inefficiently (Lü et al., 2010), resulting in an imbalance between antioxidant and oxidant levels, where oxidation is prevalent (Endres et al., 2015; Mateen et al., 2016). These overproductions can easily induce damage to proteins, nucleic acids, or lipids through free radical reactions and are associated with rheumatoid arthritis, atherosclerosis, diabetes, neurodegenerative disorders, and cancer (Burton & Jauniaux, 2011; Halliwell, 2024; Houldsworth, 2023; Mohammadian et al., 2024; Muchtaridi et al., 2024). Oxidant signaling responses to diet, microbiome, pharmaceuticals, dietary supplements, cosmetics, and environmental pollutants illustrate that redox systems respond to various environmental cues (Sies et al., 2022).

Antioxidants are essential for protecting the body from OS, as they slow or prevent oxidation, helping delay, prevent, or repair oxidative damage by clearing oxidatively damaged molecules (Halliwell, 2024). Endogenous antioxidants, including enzymes like superoxide dismutase, catalase, glutathione reductase, and glucose-6-phosphate dehydrogenase, naturally defend against OS by neutralizing free radicals and maintaining cellular redox balance. However, under certain conditions, exogenous antioxidants—such as phenolic compounds, vitamins, and probiotics—are also needed to support redox balance and reduce the oxidation of lipids, proteins,

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and DNA (Lang et al., 2024; Muchtaridi et al., 2022, 2024; Muscolo et al., 2024).

Several strains of lactic acid bacteria (LAB) produce antioxidant agents (Colares et al., 2024; Mathur et al., 2020), supporting their role in therapeutic applications and gastrointestinal disease treatment (Mohsin et al., 2021; Wieërs et al., 2020). Probiotics, as defined by the International Scientific Association of Probiotics and Prebiotics, are live microorganisms that provide health benefits when administered in adequate amounts (Hill et al., 2014). LAB are a diverse group of Gram-positive bacteria, with *Lactobacillus* and *Bifidobacterium* being the most studied (Ali et al., 2022; Feng & Wang, 2020; Suez et al., 2019). *Leuconostoc mesenteroides*, the type species of *Leuconostoc*, is notable for its resilience under gastrointestinal stress (Paula et al., 2015) and shows promise as a candidate for industrial applications due to its probiotic properties (Kumar et al., 2022).

To guarantee enough viable bacteria in the final product and effective health-promoting action in the host, it is critical to isolate strains that exhibit high viability and functionality as well as high OS resistance (Feng & Wang, 2020). To explore all the health attributes of probiotics, large-scale manipulation of microorganisms at the commercial level is a barrier that needs to be overcome (Hiremath & Viswanathan, 2022). Many elements such as carbohydrates, amino acids, peptides, vitamins, and Mg/Mn salts are required to grow LAB. For this reason, alternative media in front of commercials are optimized to reduce costs (Ficoseco et al., 2018; Ragaza et al., 2020). Cheese whey, a pollutant by-product from the dairy industry, could be an alternative for energy sources during fermentation and, at the same time, an environmental solution to solve economic problems (Capanoglu et al., 2022; Carvalho et al., 2013; Guimarães et al., 2020). For this reason, whey has been used as a medium to culture LAB and contribute to a sustainable bioeconomy (Colares et al., 2021, 2024; Iosca et al., 2023).

This work aimed to optimize the biomass production process and determine the antioxidant activity of *L. mesenteroides* M13.

1.1 Relevance of the work

Several reactive oxygen species (ROS) are related to physiological functions but also play roles in the development of certain human diseases. ROS are counterbalanced by a physiological defense network such as antioxidants. A variety of sources of exogenous antioxidants are available. We highlighted the probiotic strains that play a role in therapeutic benefits and the treatment of gastrointestinal diseases. To guarantee enough viable bacteria in the final product and effective health-promoting action in the host, it is critical to isolate strains that exhibit high viability and functionality with a high shelf-life.

2 MATERIAL AND METHODS

2.1 Microorganisms and maintenance

L. mesenteroides M13, a LAB isolated from artisanal minas cheese (Guimarães et al., 2020), was used in this study. The LAB strain was maintained in MRS broth (Sigma-Aldrich, Saint Louis, USA) with 20% glycerol at -80°C.

2.2 Culture media and growth conditions

To prepare the pre-inoculum, *L. mesenteroides* M13 was cultured in an Erlenmeyer flask containing supplemented whey medium (10 g L^{-1} peptone, 10 g L^{-1} yeast extract, 0.04 g L^{-1} $MnSO_4$, and 0.2 g L^{-1} $MgSO_4$). The medium was sterilized by autoclaving at 121°C for 20 min. Culturing was done at 37°C for 15 h without shaking. The cells were then centrifuged at 1,370 × *g* for 30 min, washed with 0.85% saline, resuspended in sterile distilled water, and adjusted to an optical density $(OD)_{600}$ of 0.2.

2.3 Deproteinization of whey

The whey's pH was adjusted to 4.5 and autoclaved at 120 °C for 20 min. Next, the serum was filtered, and its pH was adjusted to 6.3 and autoclaved again at 120 °C for 20 min.

2.4 Growth curve of L. mesenteroides *M13*

After preparing the pre-inoculum, *L. mesenteroides* M13 was inoculated into 125 mL Erlenmeyer flasks with 25 mL of medium to establish a growth curve. The flasks were incubated at 37 °C for 24 h without shaking, with samples taken every 3 h. Biomass was measured by the dry cell mass: 24 mL of fermentation broth was centrifuged at 2,800 × *g* for 20 min, washed twice with saline, and lyophilized. For colony-forming unit (CFU) mL^{-1} counts, 1 mL of the broth was serially diluted (10^{-6}, 10^{-7}, and 10^{-8}) and plated at 0, 6, 9, 15, 18, 21, and 24 h. The plates were incubated at 37 °C for 48 h.

2.5 Optimization of biomass production of L. mesenteroides *M13*

2.5.1 Selection of significant variables by a fractional factorial design

A fractional factorial design (FFD) (2^{4-1}) was performed to evaluate the effect of independent variables on biomass production by the *L. mesenteroides* M13 strain. Table 1 describes all the formulations tested.

Table 1. Experimental design of the fractional factorial design of the *L. mesenteroides* M13 strain.

Assay	Yeast extract (g L^{-1})	Peptone (g L^{-1})	Mg (g L^{-1})	Mn (g L^{-1})
1	0 (-1)	0 (-1)	0 (-1)	0 (-1)
2	10 (+ 1)	0 (-1)	0 (-1)	.08 (+ 1)
3	0 (-1)	10 (+ 1)	0 (-1)	.08 (+ 1)
4	10 (+ 1)	10 (+ 1)	0 (-1)	0 (-1)
5	0 (-1)	0 (-1)	.4 (+ 1)	.08 (+ 1)
6	10 (+ 1)	0 (-1)	.4 (+ 1)	0 (-1)
7	0 (-1)	10 (+ 1)	.4 (+ 1)	0 (-1)
8	10 (+ 1)	10 (+ 1)	.4 (+ 1)	.08 (+ 1)
9	5 (0)	5 (0)	.2 (0)	.04 (0)
10	5 (0)	5 (0)	.2 (0)	.04 (0)
11	5 (0)	5 (0)	.2 (0)	.04 (0)
12	5 (0)	5 (0)	.2 (0)	.04 (0)

Mg: magnesium sulfate; Mn: manganese sulfate.

A first-order model was used to describe the effects of the individual supplements on biomass production in response (Equation 1).

$$\text{Response} = \beta_0 + \Sigma \beta_i X_i \quad (1)$$

Where:

β_0 represents the constant;

β_i refers to the coefficient of the linear term; and

X_i corresponds to the independent variable.

2.5.2 Central composite rotatable design

A central composite rotatable design (CCRD) was used to determine the conditions that maximize biomass production by the *L. mesenteroides* M13 strain and to fit second-order models to describe them. The main factors influencing the responses, previously analyzed according to FFD, were used in the CCRD. The variables evaluated are demonstrated in Table 2.

A second-order model was used to describe the relationship between these supplements and biomass production, according to Equation 2.

$$\text{Response} = \beta_0 + \Sigma \beta_i X_i + \Sigma \beta_{ij} X_i X_j + \Sigma \beta_{ii} X_i^2 \quad (2)$$

Where:

β_0 represents the constant;

β_i and β_{ii} refer to the terms of the linear and quadratic coefficients, respectively;

β_{ij} corresponds to the interaction term; and

X_i represents the independent variable.

Table 2. Central composite rotatable design of the *L. mesenteroides* M13 strain.

Assay	Yeast extract (g L^{-1})	Mg (g L^{-1})
1	3.782486	0.202513
2	17.21751	0.202513
3	3.782486	0.697487
4	17.21751	0.697487
5	10.5	0.45
6	10.5	0.45
7	10.5	0.45
8	1	0.45
9	20	0.45
10	10.5	0.1
11	10.5	0.8
12	10.5	0.45
13	10.5	0.45
14	10.5	0.45

Mg: magnesium sulfate.

2.5.3 Validation of models

The best conditions determined in CCRD were used for new benchtop experimentation, performing production in 25 mL of the culture medium. The predictive capacity of the established models was evaluated using bias and accuracy factors (F_B and F_A, respectively) as calculated in Equations 3 and 4, respectively, described by Baranyi et al. (1999).

Validation was performed for two culture media, conditions 1 and 2: condition 1 (10.55 g L^{-1} of yeast extract, 10 g L^{-1} of peptone, and 0.16 g L^{-1} of manganese sulfate), and condition 2 (7.5 g L^{-1} of yeast extract, 2.5 g L^{-1} of peptone, and 0.08 g L^{-1} of manganese sulfate).

$$F_B = 10^{\left(\Sigma \frac{\log\left(\frac{P}{O}\right)}{n}\right)} \quad (3)$$

$$F_A = 10^{\left(\Sigma \frac{\left|\log\left(\frac{P}{O}\right)\right|}{n}\right)} \quad (4)$$

Where:

P corresponds to the predicted values;

O corresponds to the observed values; and

n represents the number of experimental data.

F_B indicates the relative mean deviation between predicted and observed values, providing the model's confidence. An F_B value of 1 indicates that the predicted and observed values fully agree. The F_A indicates how the experimental values are arranged around the predicted values, providing the model's accuracy. Acceptable F_A values are close to 1. These factors are appropriate for comparing two or more models.

2.6 Scanning electron microscopy of lyophilized cells

Lyophilized powders of the *L. mesenteroides* M13 strain grown in MRS broth and an optimized medium were fixed to a sample slide, transferred to stubs, and then platinum-plated on Leica equipment on SCD500 (LEICA, Wetzlar, Germany). The morphology of the platinum-coated samples was examined using a Helios NanoLab 650 scanning electron microscope (FEI, Oregon, USA).

2.7 Stability assay

A 1,000-mL Erlenmeyer flask containing 500 mL of the culture medium was inoculated with the *L. mesenteroides* M13 strain with an OD_{600} of 0.2. The flasks were incubated at 37 °C for 24 h. The lyophilized material was obtained from 1,000 mL of the fermented broth of each of the four strains, which were centrifuged at 1,400 × *g* for 15 min, washed twice with saline solution, and dried in a lyophilizer for 48 h. The probiotic strains were then stored at room temperature for 4 months and at 4 °C for 8 months, with the CFU g^{-1} being evaluated every month. This experiment was performed in triplicate.

2.8 Antioxidant activity

2.8.1 Scavenging 2,2-diphenyl-1-picrylhydrazyl radicals

The *L. mesenteroides* M13 culture was incubated at 37 °C for 24 h to assess antioxidant activity in two groups: (a) intact cells and (b) lysed cells. For intact cells, 10^{10} cells mL^{-1} were prepared by centrifuging at 1,400 × *g* for 15 min, washing three times with sterile distilled water, and resuspending in sterile distilled water. For lysed cells, the culture was centrifuged at 1,400 × *g* for 15 min. Cell lysis was induced using lysozyme (1 mg mL^{-1}) at 37 °C for 1 h, followed by sonication at 90 mA for 1 min in five 1-min pulses with ice bath intervals. Post-lysis, the samples were centrifuged at 1,500 × *g* for 10 min, and the supernatant was used for antioxidant testing.

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity was measured using a modified method from Li et al. (2012). A 1-mL aliquot from each group was combined with 2 mL of the DPPH solution (0.05 mM in ethanol), vortexed, and incubated in the dark at room temperature for 30 min. Absorbance was measured in triplicate at 517 nm. Intact cells were centrifuged at 1,500 × *g* for 10 min before reading to remove cells.

The antioxidant activity was evaluated per Equation 5, with negative controls (autoclaved distilled water and autoclaved distilled water with 1 mg mL^{-1} lysozyme) subtracted from the test results. The ethanolic DPPH solution (0.05 mM) served as the test control in Equation 5. Each test was performed in triplicate across 3 separate days.

$$\text{Antioxidant activity}(\%) = \left[1 - \left(\frac{\text{Sample abs}}{\text{Control abs}}\right)\right] \times 100 \qquad (5)$$

2.8.2 Resistance of intact cells to hydrogen peroxide

The method of Buchmeier et al. (1997) was used with some modifications. A pre-inoculum of the *L. mesenteroides* M13 strain was performed; it was inoculated at 1% (v/v) in MRS broth and MRS broth containing 0.4, 0.7, or 1.0 mM hydrogen peroxide (30% by weight of solution) and incubated at 37 °C for 8 h. Cell growth was measured spectrophotometrically at 600 nm. The results were presented in OD. Spectrophotometric measurements were performed in triplicates on 3 different days.

2.9 Statistical analysis

A Student's t-test ($\alpha = .05$) was used to verify the significance and adequacy of the data, FFD, and model coefficients in the CCRD. A Pareto chart ranked the importance of controlled factors based on FFD. Fisher's test (analysis of variance [ANOVA] of linear regression) assessed the statistical impact of the yeast extract, peptone, and magnesium sulfate concentrations on *L. mesenteroides* M13 biomass in the CCRD. Model quality was evaluated through the coefficient of determination (R^2), statistical significance of the regression, and model fit. Analyses were conducted using Minitab® 18 software (Minitab Inc.). For antioxidant activity, normality tests were applied to all data, with unpaired t-tests and one-way ANOVA used for two-group and multi-group analyses, respectively, with statistical significance at $p < .05$.

3 RESULTS AND DISCUSSION

Figure 1 shows the growth curve results. The highest growth of the *L. mesenteroides* M13 strain was observed at 24 h (1.73×10^{10} CFU mL^{-1}). All subsequent experiments were carried out with the bioprocess completed in 24 h.

After the bioprocess was carried out in the 12 evaluated conditions of the FFD, plating was performed to assess the cell concentration (CFU mL^{-1}) of the serial dilutions ($10^{-5}/10^{-6}/10^{-7}$). Table 3 shows the CFU mL^{-1} values.

The results were analyzed by ANOVA, with statistical significance indicated by a $p < .05$ (Table 4). The interference of each medium component and the second-order interactions of these components were analyzed. It was observed that all variables were significant. In the second-order interactions, an influence on production was observed in the interaction between all components.

Table 5 presents the FFD regression coefficients. These coefficients express the intensity and sign of the factors' effect on biomass production. From this, it can be observed that only the yeast extract and magnesium sulfate positively affect biomass production (CFU mL^{-1}). Manganese sulfate and peptone negatively influenced biomass production and were removed from the future stages of the experiment.

Based on the results obtained in the FFD, a CCRD was planned with the following decisions: (1) an upward shift of the yeast extract concentration range, and (2) an upward shift of the manganese sulfate concentration range.

The CCRD results were obtained after 24 h of cultivation with experimental units, as shown in Table 6. An ANOVA was performed, and the variables that significantly influenced the biomass of the *L. mesenteroides* M13 strain (CFU mL^{-1}) were analyzed, as well as the interference of each component in the medium and the second-order interactions (Table 7). A detailed analysis showed that for all variables, the p-value was less than 0.05, showing the influence of these medium components in producing high biomass concentrations. The second-order interactions between yeast extract and yeast extract influenced the production by presenting a $p < .05$.

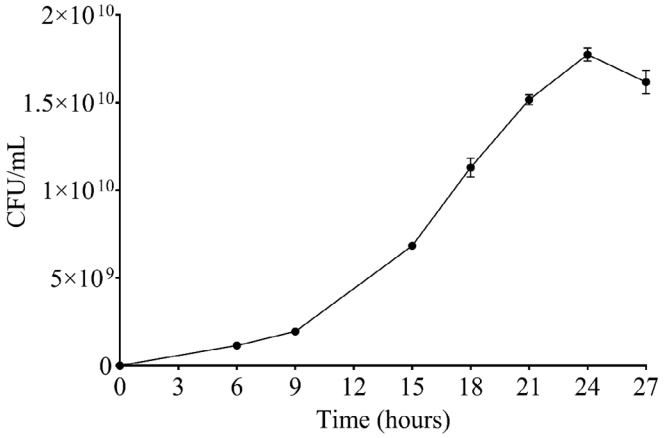

Figure 1. Growth curve of the *L. mesenteroides* M13 strain.

Response surface plots (Figure 2A) demonstrate the magnitude of factors' influence on the models. The yeast extract and magnesium sulfate positively affect biomass production, calculated as CFU mL^{-1}.

The maximum adjusted biomass production of the *L. mesenteroides* M13 strain (CFU mL^{-1}) could be achieved when values ranging from 13.67 g L^{-1} of the yeast extract and 0.73 g L^{-1} of magnesium sulfate were used (condition 1). Under this ideal condition, it was possible to produce 2.67×10^{10} CFU mL^{-1}, as shown in Figure 2B. Thus, with the whey supplementation optimization process, biomass production increased by approximately 2,542% compared to production with non-supplemented whey (1.05×10^9 CFU mL^{-1}), demonstrating success in optimization for increased productivity and cost (Table 3).

The conditions used for the validation experiment were based on the estimated values of each factor for biomass

Table 3. CFU mL^{-1} of the fractional factorial design of the *L. mesenteroides* M13 strain.

Assay	Yeast extract (g L^{-1})	Peptone (g L^{-1})	Mg (g L^{-1})	Mn (g L^{-1})	CFU mL^{-1}
1	0	0	0	0	1.05×10^9
2	10	0	0	0.08	1.10×10^{10}
3	0	10	0	0.08	8.50×10^9
4	10	10	0	0	9.80×10^9
5	0	0	0.4	0.08	1.29×10^9
6	10	0	0.4	0	2.35×10^{10}
7	0	10	0.4	0	7.60×10^9
8	10	10	0.4	0.08	1.85×10^{10}
9	5	5	0.2	0.04	1.73×10^{10}
10	5	5	0.2	0.04	1.69×10^{10}
11	5	5	0.2	0.04	1.81×10^{10}
12	5	5	0.2	0.04	1.79×10^{10}

Mg: magnesium sulfate; Mn: manganese sulfate.

Table 4. Fractional factorial analysis of the variance of the *L. mesenteroides* M13 strain.

Parameters	df	SS	MS	F-value	p-value
Model	8	5.65154×10^{20}	7.06442×10^{19}	232.89	.000
Linear	4	2.03610×10^{20}	5.09025×10^{19}	167.81	.001
Yeast extract (g L^{-1})	1	3.72100×10^{19}	3.72100×10^{19}	122.67	.002
Peptone (g L^{-1})	1	9.61000×10^{18}	9.61000×10^{18}	31.68	.011
Mg (g L^{-1})	1	1.12360×10^{20}	1.12360×10^{20}	370.42	.000
Mn (g L^{-1})	1	3.61000×10^{18}	3.61000×10^{18}	11.90	.041
Second-order interactions	3	1.12583×10^{20}	3.75277×10^{19}	123.72	.001
Yeast extract (g L^{-1})*peptone (g L^{-1})	1	4.98002×10^{19}	4.98002×10^{19}	164.18	.001
Yeast extract (g L^{-1})*Mg (g L^{-1})	1	5.97324×10^{19}	5.97324×10^{19}	196.92	.001
Yeast extract (g L^{-1})*Mn (g L^{-1})	1	3.05045×10^{18}	3.05045×10^{18}	10.06	.050
Error	3	9.10000×10^{17}	3.03333×10^{17}		
Total	11	5.66064×10^{20}			

df: degrees of freedom; SS: sum of square; MS: mean square.

Table 5. Fractional factorial regression coefficients for the *L. mesenteroides* M13 strain.

Term	Effect	Coefficient	T-value	p-value
Constant		14,150,000,000	36.66	.000
Yeast extract	12,200,000,000	6,100,000,000	11.08	.002
Peptone	-6,200,000,000	-3,100,000,000	-5.63	.011
Mg	10,600,000,000	5,300,000,000	19.25	.00
Mn	-1,900,000,000	-950,000,000	-3.45	.041
Yeast extract*peptone	-1.99600×10^{10}	-9,980,000,000	-12.81	.001
Yeast extract*Mg	10,930,000,000	5465000000	14.03	.001
Yeast extract*Mn	-2,470,000,000	-1,235,000,000	-3.17	.050
Yeast extract*peptone*Mg*Mn		7,395,000,000	21.93	.000

production, calculated by the CFU mL^{-1} of the condition. The values chosen corresponded to the desirability function (d) of 1. The experiments were conducted in triplicate, with results expressed in CFU mL^{-1}, and the values obtained for each fermentative parameter were used to calculate the F_B and F_A, which were found to be close to 1. For condition 1, the value was 1.026, indicating that the function expressed by the CCRD has accurate results, as shown in Table 8.

The scanning electron microscopy (SEM) of the lyophilized cells of the *L. mesenteroides* M13 strain grown in MRS and the optimized medium is demonstrated in Figure 3. The freeze-dried powder demonstrated a lamellar structure and no aggregate or dispersed particles (Figures 3A, 3C, 3E, and 3G), with no bareness, smooth surface, and almost no porosity. On the other hand, a granular shape and good particle dispersion were observed in the freeze-dried powders from the *L. mesenteroides* M13 strain grown in the optimized medium (Figures 3B, 3D, 3F, and 3H). Porous, long filamentous cellular structures and organic material from the cheese whey are indicated by arrows (Figures 3F and 3H).

Table 6. Results in CFU mL^{-1} of the central composite rotatable design obtained for the *L. mesenteroides* M13 strain.

Assay	CFU mL^{-1}	
	Real	Predicted
1	1.02×10^{10}	9.06×10^{9}
2	2.2×10^{10}	2.19×10^{10}
3	1.4×10^{10}	1.39×10^{10}
4	2.4×10^{10}	2.5×10^{10}
5	2.5×10^{10}	2.4×10^{10}
6	2.3×10^{10}	2.4×10^{10}
7	2.41×10^{10}	2.4×10^{10}
8	3.4×10^{9}	4.22×10^{9}
9	2.2×10^{10}	2.13×10^{10}
10	1.9×10^{10}	1.98×10^{10}
11	2.62×10^{10}	2.54×10^{10}
12	2.39×10^{10}	2.42×10^{10}
13	2.41×10^{10}	2.42×10^{10}
14	2.48×10^{10}	2.42×10^{10}

The viability of the *L. mesenteroides* M13 strain after lyophilization and storage at room temperature for 4 months and at a temperature of 4 °C for 8 months is shown in Figure 4. Initially, it was possible to verify at room temperature that the viability was already reduced by 6 log cycles in 4 months for the *L. mesenteroides* M13 strain (Figure 4A). When we analyzed the viability of the lyophilized probiotic strain at a temperature of 4 °C, less than 1 log cycle of deaths occurred in 8 months of storage (Figure 4B).

Figures 4C and 4D shows the antioxidant activity of the *L. mesenteroides* M13 strain and the resistance of intact cells to hydrogen peroxide. The percentages of capture of the radical DPPH from the supernatant and whole cells of the *L. mesenteroides* M13 strain were 81 and 65%, respectively (Figure 4C). The resistance of intact *L. mesenteroides* M13 cells to hydrogen peroxide is demonstrated in Figure 4D. The intact cells showed tolerance to hydrogen peroxide at concentrations of 0.4–1 mM for 8 h and were concentration-dependent. At the highest concentration of hydrogen peroxide (1.0 mM for 8 h), intact *L. mesenteroides* M13 cells showed an absorbance of 2.2, transforming it into a percentage of survival of the *L. mesenteroides* M13 strain under hydrogen peroxide. We found an approximate value of 86% compared to the negative control (0 mM for 8 h) (Figure 4D).

4 DISCUSSION

The use of a commercial medium to grow a probiotic strain is economically unviable since the necessity of the bioprocess is large-scale and low-cost probiotic production to deliver high numbers of bacteria (Ficoseco et al., 2018). Using some industrial by-products, such as cheese whey, has been an important strategy (Colares et al., 2021, 2024) to achieve high productivity and lower production costs. The whey is rich in nutrients, such as lactose (70%–72%), proteins (8%–10%), and mineral salts (12%–15%) (Panesar et al., 2007). Although these nutrients cannot support the growth of LAB, it is necessary to optimize the whey using additional sources of nitrogen such as yeast extract and peptone and mineral salts such as manganese sulfate and magnesium sulfate by FFD and CCRD since the concentrations

Table 7. Central composite rotatable design analysis of the variance of the *L. mesenteroides* M13 strain.

Font	df	SS	MS	*F*-value	*p*-value
Model	6	5.67184×10^{20}	9.45307×10^{19}	89.36	.000
Linear	2	3.21183×10^{20}	1.60592×10^{20}	151.80	.000
Yeast extract	1	2.89254×10^{20}	2.89254×10^{20}	273.42	.000
Mg	1	3.19294×10^{19}	3.19294×10^{19}	30.18	.001
Quadratic	2	2.45104×10^{20}	1.22552×10^{20}	115.84	.000
Yeast extract+ yeast extract	1	2.44154×10^{20}	2.44154×10^{20}	230.79	.000
Mg+ Mg	1	4.72615×10^{18}	4.72615×10^{18}	4.47	.072
Second-order interactions	1	8.10000×10^{17}	8.10000×10^{17}	0.77	.411
Yeast extract+ Mg	1	8.10000×10^{17}	8.10000×10^{17}	0.77	.411
Error	7	7.40535×10^{18}	1.05791×10^{18}		
Lack of adjustment	3	4.95202×10^{18}	1.65067×10^{18}	2.69	.181
Pure error	4	2.45333×10^{18}	6.13333×10^{17}		

df: degrees of freedom; SS: sum of square; MS: mean square.

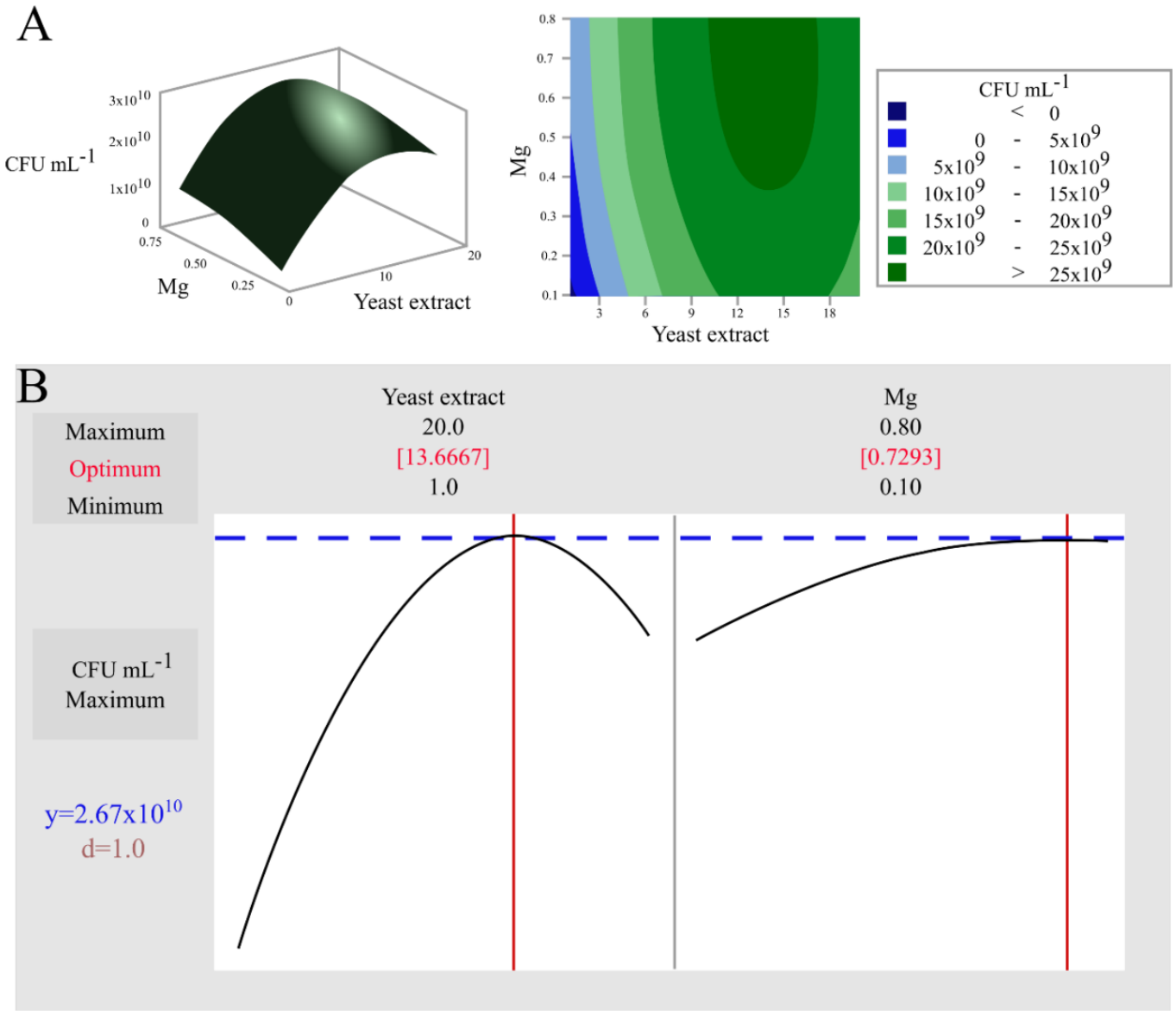

Figure 2. Response surface plot showing the interaction between experimental factors and their influence on biomass yield **(A)**. Maximum adjusted biomass production achieved under optimized conditions **(B)**.

Table 8. Validation of the central composite rotatable design for the *L. mesenteroides* M13 strain.

Strain	Condition 1		Condition 2	
	Bias factors	Accuracy factors	Bias factors	Accuracy factors
M13	1.026	1.026	.966	.966

of the protein and reducing sugar in the deproteinized whey were 3.4 and 0.6 g L^{-1}, respectively (Colares et al., 2021).

In this study, yeast extract and magnesium sulfate were necessary to grow the *L. mesenteroides* M13 strain in whey, different from what was observed for the final composition of the optimized medium for *Leuconostoc citreum* with glucose, yeast extract, K_2HPO_4, sodium acetate trihydrate, and dibasic ammonium citrate (Kim et al., 2004). The necessity of only yeast extract as a source of carbon, nitrogen, and vitamins for the growth of *L. mesenteroides* M13 strain turns the bioprocess economically viable since peptone was not necessary to complement the medium, different than observed for other LAB, such as *Lactiplantibacillus plantarum* UFSJP2 probiotic strain (Colares et al., 2024). The high concentration of amino acids and peptides in a medium could be associated with the depression of bacterial growth (Hayek et al., 2013).

LAB's growth and metabolic activity are associated with small amounts of Mn^{2+} and Mg^{2+} in the medium (Hayek et al., 2019). During the FFD study, the mineral magnesium significantly increased the *L. mesenteroides* M13 biomass when we analyzed the variation in the divalent metal ion Mn^{2+} and Mg^{2+} concentrations. LAB frequently requires divalent ions as

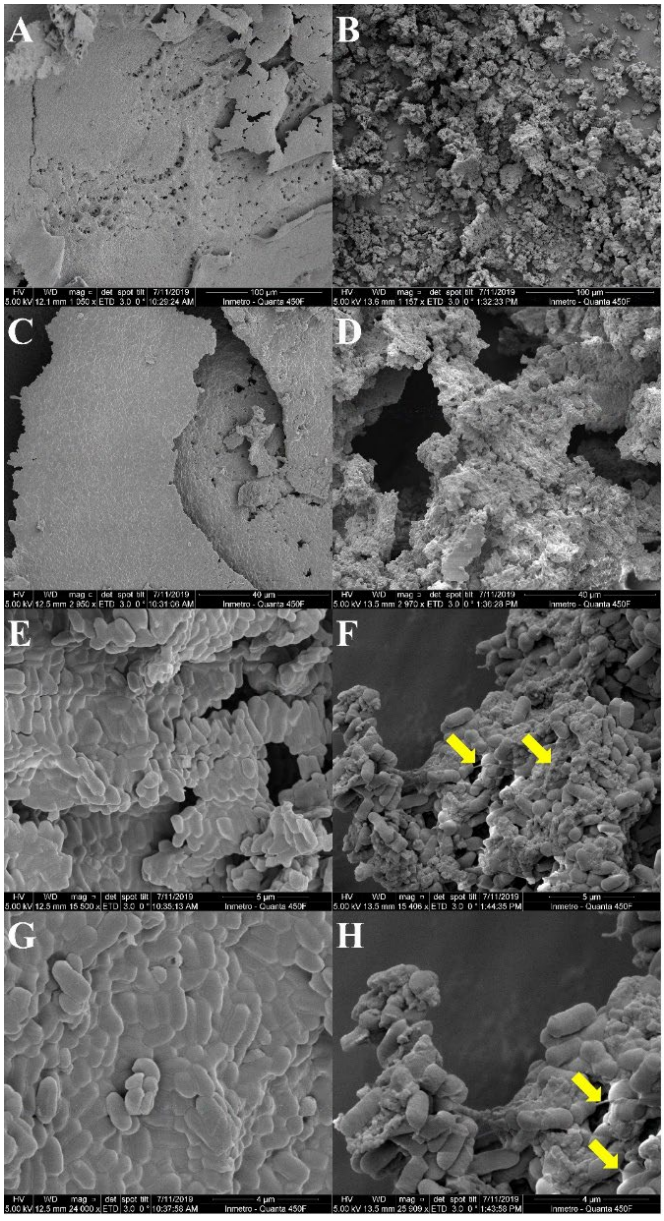

Figure 3. Scanning electron microscopy of the *L. mesenteroides* M13 strain grown in MRS **(A, C, E, and G)** and in the optimized medium **(B, D, F, and H)**. Yellow arrows indicate long, filamentous cellular structures and the organic material.

stimulatory factors for nutrition transportation and enzymatic activity (Yeboah et al., 2023). Other studies have verified the importance of metal ions and highlighted their impact on LAB microorganisms (Colares et al., 2021, 2024).

In this work, we achieved a high level of growth of the *L. mesenteroides* M13 strain, superior to 10 log CFU mL^{-1}, demonstrating a successful optimization of the bioprocess. After validating the models, the biomass production increased by 2,542% compared to the non-optimized process, which was higher than that of other studies in the literature. This result was higher than *L. citreum* biomass optimization, with 159% (Kim et al., 2004). The quantity of biomass production was higher than that of *L. mesenteroides* (8 log CFU mL^{-1}), which used a composition of the medium based on the components of plant origin as soy protein concentrate (Makowski et al., 2017).

The aggregated spherical structure observed in SEM may have a particular protective effect on the *L. mesenteroides* M13 strain grown in whey, as observed for other LAB strains (Colares et al., 2024; Wang & Zhong, 2024). Freeze-dried microcapsules of *L. plantarum* in whey protein isolate and gum Arabic coacervate maintains 59.51% viability when stored at 25°C until 60 days (Sharifi et al., 2021). The whey proteins and denatured whey proteins were demonstrated as good coating materials for the microencapsulation of probiotic bacterial *Lactobacillus plantarum* 299v strains and contributed to viability for 70 days (Sun et al., 2023). The presence of the organic material in the aggregate powder is associated with the stability of the probiotic and could be an essential strategy for microencapsulation (Wang & Zhong, 2024).

The viability during storage results was superior to other LAB strains observed in the literature. Bolla et al. (2011) demonstrated a reduction in the survival rate between .5 and 1 log cycle after 30 days of storage at 4°C using milk as a cryoprotectant between 30 and 180 days of storage; the viability of each microorganism decreased by almost 2 log cycles. *Weissella cibaria* JW15, lyophilized with cryoprotectants like lactose, achieved an immediate post-lyophilization survival rate of 71.62%–76.41% (Kim et al., 2018). The prolonged survival is due to the ability of a strain to ferment particular prebiotics and sugar and use them as fermentable carbon sources (McLaughlin et al., 2015). Some cryoprotectants are introduced to limit the adverse effects caused by low-temperature conditions in LAB strains and are used to improve long-term storage. EPS helps the *L. mesenteroides* WiKim32 strain resist cold stress, suggesting it could be used as a cryoprotective agent (Jeong et al., 2022).

High-quality diets are associated with increased levels of blood antioxidants (Halliwell, 2024). In this context, to avoid damage to cells or tissue, LAB strains have been used to decrease OS (Kim et al., 2022; Nascimento et al., 2024). The free radical scavenging activity of probiotics has been conducted to confirm the decrease in the levels of DPPH (Vougiouklaki et al., 2023). The percentage of DPPH antioxidants in this study was better than that of *L. mesenteroides* MG860 and *L. citreum* MG210 strains, with 4.06 ± 2.12 and 6.25 ± 0.86, respectively (Kim et al., 2022) and similar to what was observed for the *L. plantarum* UFSJP2 strain that presented an antioxidant activity of 79 and 46%, respectively, for intact and lysed cells (Colares et al., 2024). A significant decrease in the DPPH free radical scavenging capacity was promoted when removing these cell surface compounds by lyzed cells, as observed in the decreased antioxidant activity to 44.31% at 10^{10} CFU mL^{-1} for *L. plantarum* C88 (Li et al., 2012). Proteins or polysaccharides as products from the cell surface are related to the antioxidant activity of some LAB strains (Hoffmann et al., 2021), and studies have demonstrated the importance of some enzymes, such as superoxide dismutase, in the antioxidant activity of LAB (Spyropoulos et al., 2011). Probiotic strains have been found to resist hydrogen peroxide. In the study of *L. plantarum* UFSJP2, the strain exhibited approximately 77.5% survival under hydrogen

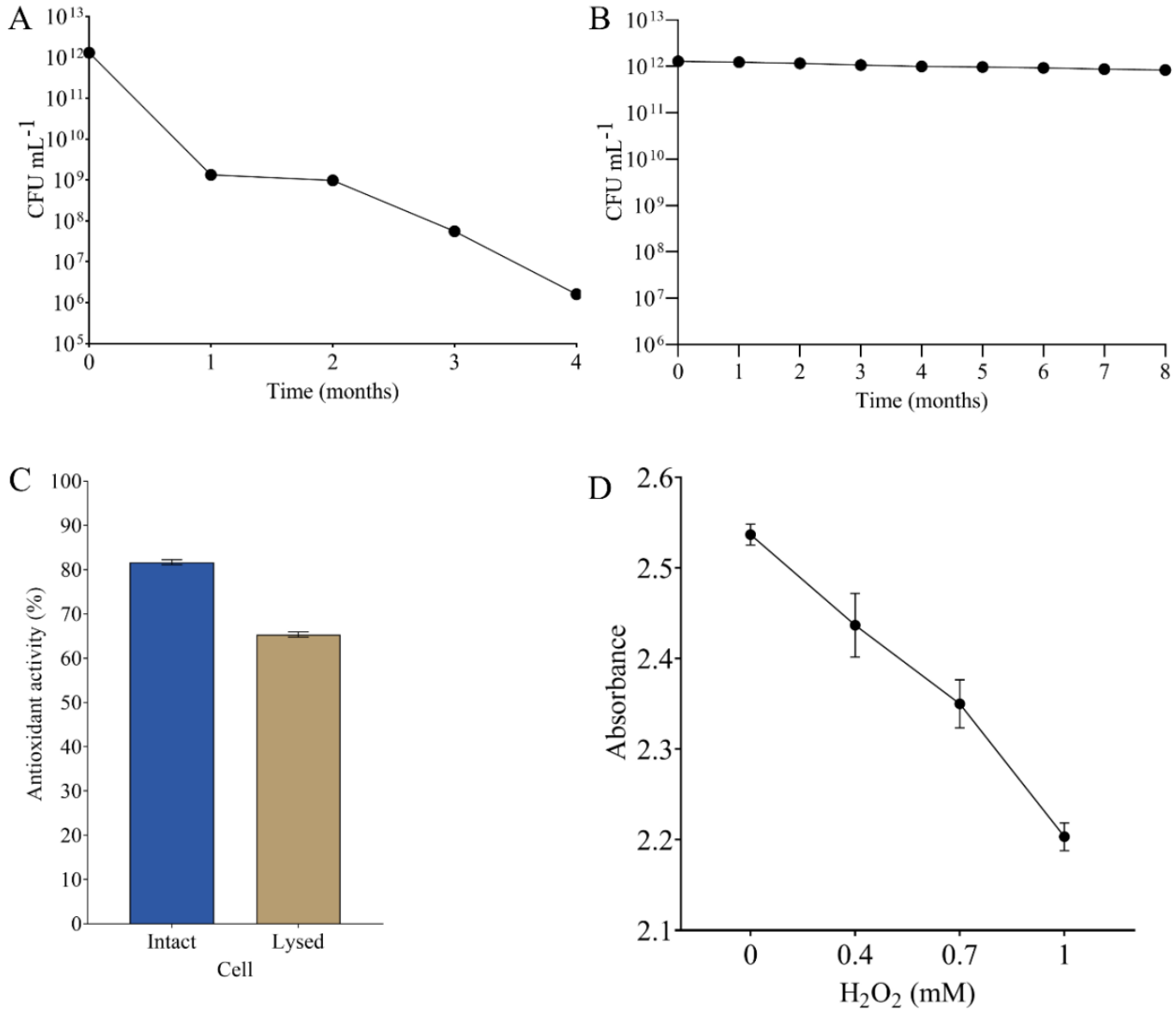

Figure 4. Viability of the *L. mesenteroides* M13 strain following lyophilization and storage for 4 months at room temperature **(A)** and 8 months at 4°C **(B)**. The antioxidant activity of the *L. mesenteroides* M13 strain **(C)**. The resistance of intact *L. mesenteroides* M13 cells to hydrogen peroxide **(D)**.

peroxide exposure compared to the negative control (0 mM for 8 h) (Colares et al., 2024). On the other hand, no resistance to hydrogen peroxide was observed for the *Lactobacillus casei* 01 strain (Lee et al., 2005).

5 CONCLUSION

The use of FFD and CCRD to study the components of the supplementation of the whey for the *L. mesenteroides* M13-optimized bioprocess presented an approximately 2,542% higher yield when compared to whey without supplementation and biomass production of 2.67×10^{10} CFU mL^{-1}, making the bioprocess economically viable and sustainable, contributing to the bioeconomy. The *L. mesenteroides* M13 strain demonstrated antioxidant activity and could be added to diverse food matrices to promote benefits for human health. The viability of the lyophilized strain storage at 4°C was maintained for up to 8 months. The *L. mesenteroides* M13 strain demonstrated a valuable supplement to application in the food industry.

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