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Research Article

Response Surface Methodology Mediated Optimization of Medium Components for Growth Density and Rate of *Mycoplasma gallisepticum*

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Abstract

This study aimed to optimize the composition of a liquid medium for *Mycoplasma gallisepticum* growth rate and density through statistical approach. The growth concentration of *M. gallisepticum* was determined by plotting a standard curve using Real-Time Quantitative PCR. The one-factor-at-a-time method and the Plakett-Burman design were initially applied to identify the factors that influenced the biomass of *M. gallisepticum*. The steepest ascent experiment and response surface methodology (RSM) with Box-Behnken design was employed to simultaneously assess the effects of multiple factors. Finally, optimization of the initial pH and validation of the model were done. The optimum concentrations of the critical components were obtained as follows: 203.96 mL/L of horse serum, 9.64 g/L of glucose, and 9.49 g/L of PPLO broth. The nucleic acid copy number of *M. gallisepticum* reached $10^{10.5147}$ copies/mL and the viable cell count reached a maximum of $10^{9.8451}$ CCU/mL. This medium reduced the incubation time by approximately 6 h, and *M. gallisepticum* nucleic acid concentration and viable cell count were higher than those in the modified Frey medium (9.99 and 7 times, respectively). The new liquid medium is likely to improve productivity and reduce the production costs for vaccine-manufacturing companies in the future by reducing incubation times and increasing the growth rate and concentration of *M. gallisepticum*.

Keywords: Body weight, Continuous light stress, Cortisol, Cystic follicle, *Ferula*, Ovary, Oxidative stress

Introduction

Mycoplasma gallisepticum is a significant pathogen in poultry and wild birds [1]. *M. gallisepticum* can cause Chronic Respiratory Disease (CRD) in chickens [2], which results in high economic losses because of reduced feed conversion efficiency, slow growth, and decreased hatchability [3]. Both vertical and horizontal transmission of the pathogen is possible, and *M. gallisepticum* can also cause coinfections with other respiratory pathogens, which increases the mortality rate [4-6]. When chickens are infected with *M. gallisepticum*, antibiotics are commonly used to control the infection. However, completely clearing *M. gallisepticum* from infected chickens is impossible, and the concerns of increased drug resistance and antibiotic residues cannot be ignored [7,8].

Inoculating chickens with the *M. gallisepticum* vaccine is regarded as an acceptable method for providing protection [9]. Presently, these vaccines are primarily produced via microbial fermentation, the medium composition and the culture concentration of high-density fermentation culture are the critical factors that impact the cost of the vaccine [10]. It is uneasy to culture *M. gallisepticum* as it has limited synthesis capacity; therefore, it requires large amounts of nutrients from the external environment [11,12]. The most commonly used culture component in the *Mycoplasma* medium is based on pleuropneumonia-like organisms (PPLO) and is widely utilized [13]. The composition of the medium (carbon and nitrogen sources) is a crucial element influencing biological growth as fermentation conditions.

Hence, optimizing the medium and the fermentation conditions can aid in increasing biomass and reducing costs [14,15]. Response surface methodology (RSM) is an empirical statistical technique that uses mathematical modeling to determine the mutual effects of various process parameters on the response variable. Quantitative data generated from the design of experiments and the analysis of regression models and operational conditions can result in high-end performance [16]. Several statistically-based experimental designs have been used to optimize the fermentation medium, and these include full factorial design, partial factorial design, Plackett-Burman design (PBD), Box-Behnken design (BBD), and central composite design (CCD) [17]. Currently, PBD and RSM are successfully used to optimize the variables in the culture medium. These methods are used to enhance the production of industrially important metabolites by various microorganisms such as fungi, bacteria, and actinomycetes [18-20]. The concentration of viable bacterial cells of *Lactobacillus plantarum* Y44 cultured at 37°C for 16 h in the optimized medium was 3.363×10^{10} CFU/mL. This amount was 6.11 times higher than that in the MRS medium [21]. The yield of valine was 457.23±9.52 mg/L after optimizing the fermentation medium of *Streptomyces* sp. Zjut-iF-354 using RSM, which is the highest yield ever reported [22].

In this study, the *M. gallisepticum* R strain, which was preserved via lyophilization in our laboratory, was used for fermentation. Carbon and nitrogen sources that can increase the concentration of *M. gallisepticum* were screened using the one-factor-at-a-time method. Subsequently, the primary factors that affect the concentration of *M. gallisepticum* were optimized via RSM. Furthermore, a fermentation medium suitable for industrial production was screened and verified experimentally, which laid the foundation for further industrial application.

Material and Methods

Mycoplasma gallisepticum R Strain and Culture Medium

Mycoplasma gallisepticum R strain, which was freeze-dried and preserved by Shandong Lvdu Biotechnology Co., LTD. (Shandong, China), was used as the research object and was passaged at least three times before use. NaCl, KCl, $MgSO_4$·7H2O, Na_2HPO_4·12H_2O, KH_2PO_4, glucose, L-cysteine solution, arginine solution, penicillin, phenol red, and trehalose were bought from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China), yeast extract and yeast peptone was bought from Angel Yeast Co., Ltd. (Hubei, China), lactalbumin hydrolysate, peptone, heart infusion broth, beef extract, brain heart infusion, trypticase soy broth, and PPLO broth was bought from ThermoFisher Scientific (Waltham, MA, USA), NADH I was bought from Beijing Ruitaibio Co., Ltd. (Beijing, China), horse serum was bought from Tianjin Kangyuan Biotechnology Co., Ltd. (Tianjin, China).

Modified Frey medium: 5.0 g of NaCl, 0.4 g of KCl, 0.2 g of $MgSO_4$·7H2O, 1.6 g of Na_2HPO_4·12H_2O, 0.2 g of KH_2PO_4, 10.0 g of glucose, 5.0 g of lactalbumin hydrolysate, 5.0 g of yeast extract, 10 mL of 1% NADH I, 10 mL of 1% L-cysteine solution, 20 mL of 2% arginine solution, 10 mL of penicillin 80.000 U/mL, 1 mL of 1% phenol red, 100 mL of horse serum were added into 1000 mL sterile water. These ingredients were mixed and dissolved, the initial pH was adjusted to 7.6-7.8, and bacteria were removed via filtration through a 0.22-μm syringe filter (Guangzhou Jet Biofiltration Co., Ltd., China).

Fermentation Conditions for the *Mycoplasma gallisepticum* R Strain

This culture was inoculated aseptically (10% v/v) into 20 mL medium contained in a 100-mL glass bottle, incubated at 37°C until an orange-yellow color was obtained.

Measurement of the Nucleic Acid Concentration of *Mycoplasma gallisepticum*

Real-time quantitative PCR was performed to determine the nucleic acid concentration of *M. gallisepticum*. The standard curve was established according to the different concentrations of nucleic acid standard of *M. gallisepticum* and Ct value.

Sample Preparation

The fermentation medium cultured was heated at 95°C for 10-15 min and diluted 10 times for Real-Time Quantitative PCR detection.

Determination of Medium Composition Using the One-Factor-At-a-Time Method

Adequate nutrients are present in the culture medium of *M. gallisepticum*. Carbon and nitrogen sources and serum are essential for the growth of *M. gallisepticum*. To obtain a higher concentration of *M. gallisepticum*, eight basic media were configured, and the sources of carbon source (3 g/L of trehalose) and nitrogen sources (10 g/L of peptone, yeast peptone, heart infusion broth, beef extract, brain heart infusion, trypticase soy broth, PPLO broth) with different compositions were supplemented after the nitrogen source in the modified Frey medium was reduced to 50%.

Plackett-Burman Design (PBD)

The PBD was used to screen for and evaluate variables that exerted significant effects on the concentration of *M. gallisepticum*, but the results did not describe the interactions between these variables. According to the preliminary screening results, seven variables were selected, namely, brain heart infusion (X_1), yeast extract (X_2), lactalbumin hydrolysate (X_3), trypticase soy broth

Table 1. Plackett-Burman design factors and levels			
Factors	**Variable**	**Level**	
		Low Level (-1)	**High Level (1)**
X_1	Brain Heart Infusion	10	20
X_2	Yeast Extract	2.5	5
X_3	Lactalbumin Hydrolysate	2.5	5
X_4	Trypticase Soy Broth	10	20
X_5	Horse Serum	100	200
X_6	Glucose	10	20
X_7	PPLO Broth	10	20

(X_4), horse serum (X_5), glucose (X_6), and PPLO broth (X_7). The high and low levels of each factor were selected. The variables represented by high and low levels were coded as +1 and −1, respectively; the concentration of components in the initial medium was set to the low level (−1), and the high level was set to twice the low level. The factors and levels are listed in *Table 1*.

The Steepest Ascent Experiment

The steepest ascent experiment was designed based on the results of PBD. The appropriate direction and change steps were determined based on the influence values of each factor. According to the results of PBD, the direction and change steps of the steepest ascent experiment were estimated. The highest concentration of *M. gallisepticum* was rapidly approached based on the three main influencing factors, namely horse serum, glucose, and PPLO broth.

Box-Behenken Design (BBD)

BBD based on response surface analysis is a statistical-mathematical method that reflects the best matching conditions obtained when factors in a multifactorial system interact with each other to achieve the maximum response value. Based on the results of PBD, three factors (horse serum, glucose, and PPLO broth) at three levels (−1, 0, and 1) were selected for the next test, and Real-Time Quantitative PCR was used to determine the concentration of *M. gallisepticum* nucleic acid. The factors and levels in the experimental program are presented in *Table 2*.

Effect of Medium pH on Concentration

The effect of the initial pH of the culture medium on concentration was investigated to determine the optimal culture conditions for the *M. gallisepticum* R strain. The pH was adjusted using 1M NaOH (7.4, 7.6, 7.8, and 8.0) before filtration and incubated until an orange-yellow color was obtained. Subsequently, Real-Time Quantitative PCR was used to determine optimal fermentation conditions and provide process parameters for fermentation and cultivation in large-volume fermenters.

Table 2. Response surface experimental factors and levels				
Name	**Factors**	**Level**		
		-1	**0**	**1**
X_5	Horse Serum	200	205	210
X_6	Glucose	9	9.5	10
X_7	PPLO Broth	9	9.5	10

Model Validation

The optimized fermentation conditions were subjected to a model validation fermentation using response surface analysis. The concentration of *M. gallisepticum* was then determined using Real-Time Quantitative PCR and the color change unit (CCU) at the end of the fermentation, and the response values were compared with the predicted data.

Statistical Analysis

All experiments were repeated three times, and the data were presented as mean ±SD. The data were analyzed using one-way ANOVA and Duncan's post-hoc pairwise multiple range test to determine the magnitude of differences, and differences between sample mean values of $P<0.05$ were considered significant. The quality of fit for the polynomial model equations was quantified using the determination coefficient (R^2). The PBD and BBD data were analyzed using the Design Expert software (Version 10.0.3., USA), and GraphPad Prism 7.0 (GraphPad Software, USA) was used to perform all analyses.

Results

Rapid Detection Method for Nucleic Acid Concentration

Based on research previously conducted in our laboratory, a standard curve was plotted by correlating Real-Time Quantitative PCR Ct values with the copy number of *M. gallisepticum* nucleic acid samples *(Fig. 1)*. The equation of the standard curve was y = −3.646x+44.208, with a correlation coefficient R^2 of 0.998. It was utilized to

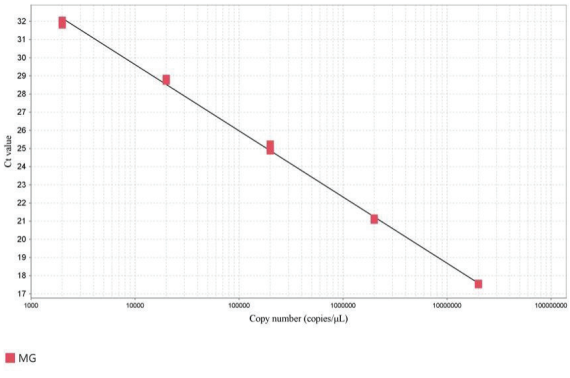

Fig 1. Standard curve of nucleic acid copy number of *M. gallisepticum* (MG: *Mycoplasma gallisepticum)*

rapidly identify the concentration of *M. gallisepticum* in the following fermentation process.

Effect of Different Nutrient Sources on the Concentration of *M. gallisepticum*

Nutrient requirements are crucial during the fermentation of *M. gallisepticum*. The findings demonstrated that different nutrients had a significant effect on the growth of *M. gallisepticum* (*Table 3*). The concentration of *M. gallisepticum* increased to $10^{10.1998}$ copies/mL when the PPLO broth was added; however, the addition of a carbon source (trehalose) did not have any effect (P>0.05) on the concentration of *M. gallisepticum*. Therefore, the three best nitrogen sources (brain heart infusion, trypticase soy broth, and PPLO broth) that increased the concentration of *M. gallisepticum* were selected for the subsequent experiments.

PBD to Screen the Main Effect Factors

To determine the factors with the highest influence on the concentration of *M. gallisepticum*, 12 different media were designed and formulated for testing using PBD. The observed responses are presented in *Table 4*, and the concentration of *M. gallisepticum* nucleic acid was found to vary from $10^{7.8143}$ copies/mL to $10^{10.2333}$ copies/mL (*Table 4*).

Regression analysis and ANOVA of the PBD were performed, as shown in *Table 4*. The regression was found to be significant (P-value 0.0459), and horse serum, glucose, and PPLO broth exhibited P-values of 0.0300, 0.0185, and 0.0359, respectively (*Table 5*). Prob>F values <0.05 denote that the model term holds significance. Thus, X_5, X_6, and X_7 were identified to be significant model terms and were deemed to be the variables with the highest effect on the nucleic acid concentration of *M. gallisepticum*. The

Table 3. *Results of the one-factor-a-time method*

Factors	Components	Copy Number (log copies/mL)
Unoptimized medium		9.3748
Nitrogen sources	Peptone	9.6181
	Yeast Peptone	9.8101
	Heart Infusion Broth	9.8126
	Beef Extract	9.8201
	Brain Heart Infusion	9.8439
	Trypticase Soy Broth	9.9114
	PPLO Broth	10.1998
Carbon sources	Trehalose	9.3217

Table 4. *Results of the Plackett-Burman design*

Run	Code Number							Copy Number (log copies/mL)
	X_1	X_2	X_3	X_4	X_5	X_6	X_7	
1	-1	-1	-1	-1	-1	-1	-1	10.1656
2	1	-1	1	1	-1	1	1	8.2194
3	1	1	-1	1	1	1	-1	8.7726
4	-1	1	1	1	-1	-1	-1	10.1209
5	1	-1	1	1	1	-1	-1	10.0577
6	1	-1	-1	-1	1	-1	1	9.7113
7	1	1	-1	-1	-1	1	-1	8.3793
8	1	1	1	-1	-1	-1	1	8.2723
9	-1	-1	1	-1	1	1	-1	10.2333
10	-1	-1	-1	1	-1	1	1	7.8143
11	-1	1	-1	1	1	-1	1	9.9415
12	-1	1	1	-1	1	1	1	9.1540

Table 5. *Analysis of the variance of PBD*

Source	Factors	Freedom	Coefficient Estimate	Sum of Squares	Mean Square	F-Value	P-Value
Model		7	9.24	8.24	1.18	6.41	0.0459*
X_1	Brain Heart Infusion	1	-0.33	1.34	1.34	7.32	0.0538
X_2	Yeast Extract	1	-0.13	0.20	0.20	1.10	0.3525
X_3	Lactalbumin Hydrolysate	1	0.11	0.14	0.14	0.73	0.4397
X_4	Trypticase Soy Broth	1	-0.082	0.082	0.082	0.44	0.5417
X_5	Horse Serum	1	0.41	2.00	2.00	10.88	0.0300*
X_6	Glucose	1	-0.47	2.70	2.70	14.71	0.0185*
X_7	PPLO Broth	1	-0.38	1.78	1.78	9.66	0.0359*

*R^2 = 91.81%; * Significant difference at P<0.05*

Table 6. *The design and results of the steepest ascent experiment*

Run	X_5 (mL/L)	X_6 (g/L)	X_7 (g/L)	Copy Number (log copies/mL)
1	200	10	10	10.4284
2	205	9.5	9.5	10.4973
3	210	9	9	10.4371
4	215	8.5	8.5	10.3987
5	220	8	8	10.3580

remaining variables exerted minimal and mathematically insignificant effects (P>0.05), as depicted in *Table 5*. Furthermore, the correlation coefficients for horse serum, glucose, and PPLO broth were 0.41, −0.47, and −0.38, respectively, which indicated that horse serum exhibited a positive impact and that glucose and PPLO broth exerted negative effects *(Table 5)*. Thus, X_5, X_6, and X_7 were selected for further optimization studies. In the next optimization step, the concentrations of horse serum, glucose, and PPLO broth in the fermentation liquid medium were increased.

The Steepest Ascent Experiment

To better approximate the domain of maximum response values for each major factor in the subsequent response surface analysis, a steepest ascent experimental design was utilized. The concentrations of horse serum, glucose, and PPLO broth were varied by 5 mL/L, 0.5 g/L, and 0.5 g/L, respectively, in the steepest ascent test, and the response values for the steepest ascent experimental design are presented in *Table 6*.

The nucleic acid concentration of *M. gallisepticum* exhibited a pattern of increase, which was followed by a decrease; hence, the design was reliable. During the second experiment, the concentration peaked at $10^{10.4973}$ copies/mL, which represented the maximum response value across all three factors. So, this point was selected to set the base concentration of BBD.

Optimization Using the BBD

Nutrient requirements are crucial during the fermentation of *M. gallisepticum*. Therefore, optimizing the nutrient composition of the medium is an effective way to increase its concentration. Response surface analysis is an effective approach to optimize fermentation parameters. This study was performed to assess the interaction of primary variables, such as X_5 (horse serum), X_6 (glucose), and X_7 (PPLO broth), using BBD to enhance the concentration of *M. gallisepticum*. The experimental design and obtained results are displayed in *Table 7*. The regression equation was obtained as follows: $Y = 10.49 - 0.1X_5 + 0.046X_6 - 0.025X_7 + 0.038X_5X_6 - 0.1X_5X_7 - (7.825E-003)X_6X_7 - 0.21X_5^2 - 0.071X_6^2 - 0.11X_7^2$

The statistical significance of the model equations was evaluated using the F-test for ANOVA, and the results are presented in *Table 7*. The ANOVA regression model showed a determination coefficient (R^2) of 0.9845, which indicated that the model accounted for 98.45% of the variance in the response *(Table 8)*. The adjusted coefficient of determination (R^2adj) was 0.9647 *(Table 8)*, and the high value implied the strong significance of the model. In this model, P<0.05, which signified that the regression was significant. Lack of fit (P>0.05) was not significant, which denoted that the model had a high level of confidence and could predict changes in the nucleic acid concentration of *M. gallisepticum (Table 8)*.

Table 7. *The results of the Box-Behenken design*

Run	Code Number			Copy Number (log copies/mL)
	X_5	X_6	X_7	
1	0	0	0	10.5092
2	-1	-1	0	10.3084
3	-1	0	-1	10.1945
4	1	0	-1	10.1833
5	0	0	0	10.4831
6	0	-1	-1	10.2473
7	0	0	0	10.5037
8	0	-1	1	10.2189
9	0	1	-1	10.4042
10	1	-1	0	10.0536
11	0	1	1	10.3445
12	-1	1	0	10.2769
13	0	0	0	10.4723
14	-1	0	1	10.3468
15	1	1	0	10.1732
16	1	0	1	9.9191
17	0	0	0	10.4688

Table 8. *The experimental results of the Box-Behenken design*

Source	Sum of Squares	Degree of Freedom	Mean Square	F-Value	P-Value
Model	0.44	9	0.049	49.52	< 0.0001*
X_5	0.079	1	0.079	80.21	< 0.0001*
X_6	0.017	1	0.017	17.33	0.0042*
X_7	5.000E-003	1	5.000E-003	5.05	0.0595
X_5X_6	5.708E-003	1	5.708E-003	5.76	0.0475*
X_5X_7	0.043	1	0.043	43.77	0.0003*
X_6X_7	2.449E-004	1	2.449E-004	0.25	0.6343
X_5^2	0.19	1	0.19	193.87	< 0.0001*
X_6^2	0.021	1	0.021	21.30	0.0024*
X_7^2	0.054	1	0.054	54.16	0.0002*
Residual	6.936E-003	7	9.909E-004		
Lack of Fit	5.603E-003	3	1.868E-003	5.60	0.0647
Pure Error	1.333E-003	4	3.333E-004		
Cor Total	0.45	16			

*R^2 = 0.9845; R^2 adj = 0.9647; * Significant difference at P<0.05*

The optimal level of interaction between any two variables was visualized using the 3D response surface. The 3D response surface plots illustrated the correlation among horse serum, glucose, and PPLO broth *(Fig. 2-A,B,C)*. According to the response surface analysis plots, the term X_5X_6 and X_5X_7 exerted a significant effect (P<0.05) on *M. gallisepticum* nucleic acid concentration *(Fig. 2-D,E)*. However, X_6X_7 did not have significant effects (P>0.05) on nucleic acid concentration of *M. gallisepticum* *(Fig. 2-F)*. At horse serum concentrations of 200-203.96 mL/L, the nucleic acid concentration of *M. gallisepticum* increased, but it decreased at concentrations 203.96-210 mL/L *(Fig.*

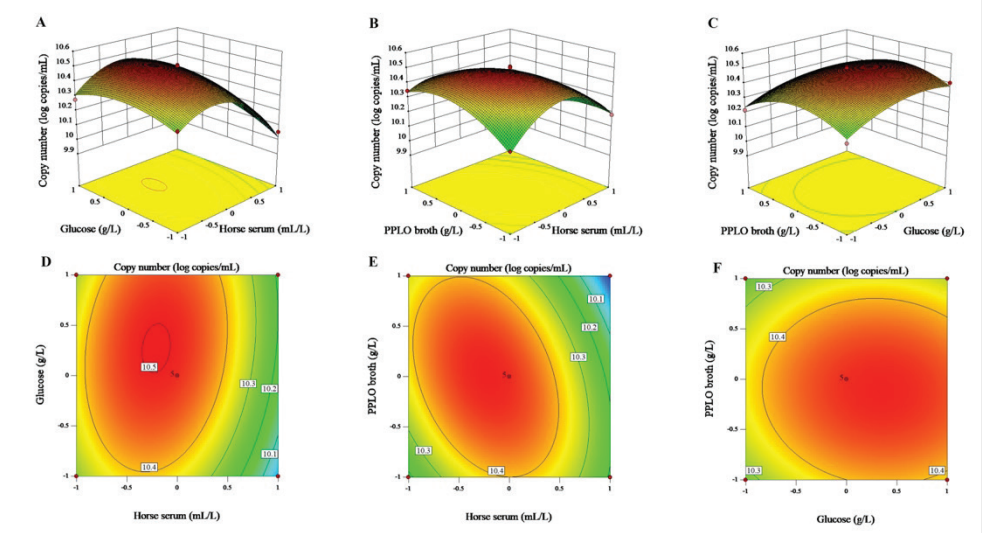

Fig 2. Response surface plots of horse serum (mL/L), glucose (g/L), and PPLO broth (g/L) on *M. gallisepticum* nucleic acid concentration. **(A)** The effect of horse serum and glucose levels, **(B)** The effect of horse serum and PPLO broth levels, **(C)** The effect of glucose and PPLO broth levels, **(D)** Horse serum and glucose levels, **(E)** Horse serum and PPLO broth levels, **(F)** Glucose and PPLO broth levels

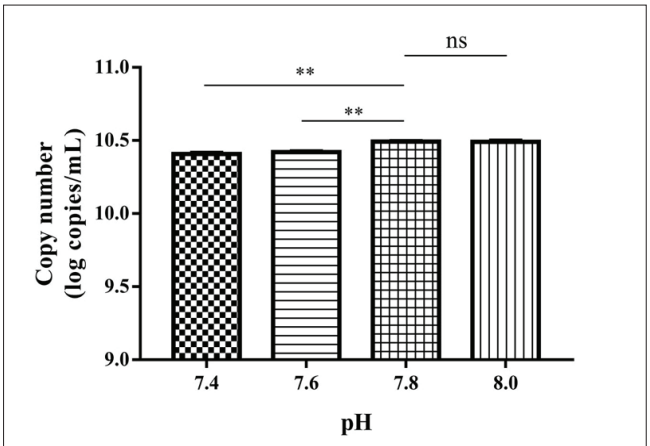

Fig 3. Nucleic acid copy number of *M. gallisepticum* under various pH values (*P<0.05, **P<0.01)

2-A,D). With an increase in the concentrations of glucose (9.00-9.64 g/L) and PPLO broth (9.00-9.49 g/L), the nucleic acid concentration increased and then dropped. When the concentrations of horse serum, glucose, and PPLO broth were 203.96 mL/L, 9.64 g/L, and 9.49 g/L, respectively, the regression model predicted a maximum value of $10^{10.504}$ copies/mL.

Effects of pH of the Medium on Nucleic Acid Concentration of *M. gallisepticum*

The nitrogen source and the pH value affected the growth of *M. gallisepticum* during fermentation in the medium. The growth of *M. gallisepticum* in the optimized medium was investigated to ascertain its response to the initial pH. As shown in *Fig. 3*, the results implied that the initial pH of 7.8 resulted in the maximum nucleic acid concentration of $10^{10.492}$ copies/mL, which was not significant at pH 8.0 (P>0.05). The maximum concentration of *M. gallisepticum* was attained when the initial pH was 7.8-8.0. Lower pH led to a decrease in the nucleic acid concentration of *M. gallisepticum*, which indicated a negative impact on its growth.

Validation of the Optimized Medium

Under the optimized conditions, the nucleic acid copy number of *M. gallisepticum* reached $10^{10.5147}$ copies/mL, which was close to the predicted value of RSM, thereby indicating that the experimental values agreed well with the predicted values *(Fig. 4-A)*. Compared with the unoptimized medium, the nucleic acid concentration of *M. gallisepticum* was increased by 9.99 times in the optimized medium *(Fig. 4-A)*. Furthermore, the optimized medium

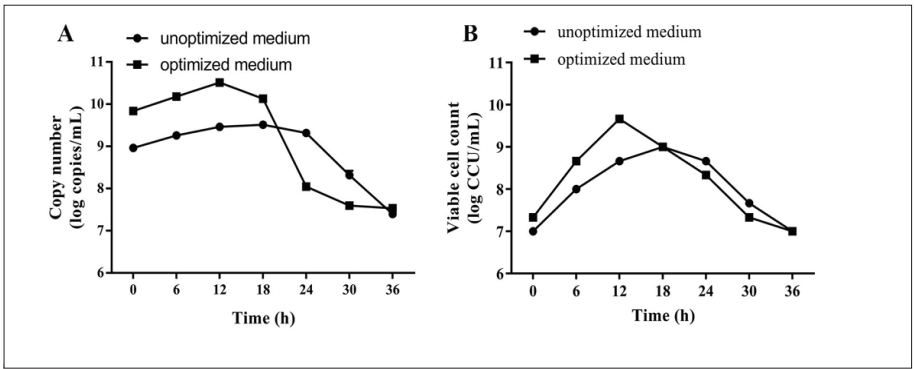

Fig 4. Growth curves of *M. gallisepticum.* **(A)** *M. gallisepticum* nucleic acid copy number growth curve, **(B)** *M. gallisepticum* CCU growth curve

exerted an effect on the viable cell count, which was up to $10^{9.8451}$ CCU/mL, signifying a 7-fold increase in the viable cell count compared with the unoptimized medium *(Fig. 4-B)*. The optimized medium enabled *M. gallisepticum* to reach the logarithmic growth stage earlier, which was approximately 6 h earlier compared with the unoptimized medium. The two growth curves demonstrated a rapid decline in nucleic acid concentration and viable cell count after *M. gallisepticum* reached maximum growth *(Fig. 4-A,B)*.

Discussion

Color change unit (CCU) is the conventional quantitative detection method for *M. gallisepticum* and this culturing step is time-consuming and difficult. In this context, Real-Time Quantitative PCR can be employed for the rapid determination of the concentration of *M. gallisepticum* [23]. This method serves as a time-efficient alternative to conventional quantitative assays, does not require further incubation, and accelerates the process of optimization. Therefore, this technique was used to rapidly determine the concentration of *M. gallisepticum*.

The addition of different nitrogen sources can increase the growth concentration of *M. gallisepticum*. Therefore, optimizing the nutrient composition of the medium is an effective way to increase the concentration of the bacterium. Response surface analysis is an effective approach to optimize fermentation parameters. PPLO broth, tryptic soy broth, and brain heart infusion media have been used for the cultivation of microorganisms, *and* PPLO broth improved both the quantity and quality of the harvested Apx toxins of *Actinobacillus pleuropneumoniae* [24]. Hwang [25] performed an experiment using CCD-optimized conditions and obtained 2.96 mg/L of *Mycoplasma* proteins, which was a three-fold increase over the unoptimized medium.

In the present study, the optimal carbon and nitrogen sources were determined using a unidirectional test, the main factors affecting *M. gallisepticum* growth were screened via PBD testing, and the fermentation parameters that resulted in the highest nucleic acid concentration were analyzed using a response surface analysis method. Under the optimized fermentation conditions, the nucleic acid copy number of *M. gallisepticum* reached $10^{10.5147}$ copies/mL, which was comparable to the predicted value. Therefore, the concentration of *M. gallisepticum* can be optimized using the RSM. The nitrogen source and the pH value affected the growth of *M. gallisepticum* during fermentation in the medium. The maximum concentration of *M. gallisepticum* was attained when the initial pH was 7.8-8.0. The growth curve of *M. gallisepticum* showed 9.99 times higher nucleic acid concentration and 7 times more viable cell count when compared with the unoptimized medium, which lowered the production cost. The logarithmic phase of *M. gallisepticum* was increased by approximately 6 h, which led to a 33.33% higher production efficiency. The new medium not only augmented the concentration but also shortened the fermentation time of *M. gallisepticum*. Nonetheless, attention must be paid to the harvesting time to avoid overculturing. Although the new medium increased the concentration of *M. gallisepticum*, serum costs remained high. Media containing 5% egg yolk, 10% horse serum, and 10% porcine serum all resulted in *M. gallisepticum* concentrations of up to 10^9 CCU/mL by the 3rd day, which could be used instead of serum. However, the number of viable organisms declined rapidly after the highest concentration was attained [26], which is in line with the results of the present study. Optimal lipid and albumin conditions established for *M. gallisepticum* were then used to propagate five different *Mycoplasma* spp. to growth levels that either equaled or surpassed those obtained with a medium containing 17% fetal bovine serum [27]. Lin [28] reported that the pH values of *M. gallisepticum* and *Mycoplasma synoviae* growth media were readjusted back to the original alkaline state when the pH reached 6.1 *(M. gallisepticum)* and 6.7 *(M. synoviae)*, and the medium was reincubated until the pH returned to 6.7-6.9. The *M. gallisepticum* and *M. synoviae* antigen yields were 43%

and 54% higher than those obtained at the usual harvest time. In the future, the concentration of *M. gallisepticum* could be increased and the cost of its medium could be reduced by adding supplements to replace the serum or by streaming NaOH solution. The cost of immunization for the farming industry could therefore be reduced.

In conclusion, this study optimized the fermentation medium for *M. gallisepticum* culture so that the nucleic acid copy number of *M. gallisepticum* reached $10^{10.5147}$ copies/mL. Compared with the unoptimized medium, the *M. gallisepticum* nucleic acid concentration in the optimized medium was increased by 9.99 times. Moreover, the viable cell count reached a maximum of $10^{9.8451}$ CCU/mL, which was a 7-fold increase compared with the unoptimized medium. The optimized medium may allow *M. gallisepticum* to reach the logarithmic growth phase earlier than the unoptimized medium, which could provide a basis for large-scale fermentation and cultivation for vaccine enterprises.

Declarations

Availability of Data and Materials: The datasets generated and/or analysed during the study are available from the corresponding authors and can be provided upon request.

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Authors' Contributions: YS performed the experiments, analysed the results, and drafted the manuscript. XZ and JZ assisted in the experimental design and summarized the experimental results. YY and XX put forward valuable suggestions for the revision and improvement of the paper. ZS, SL and LC conceived and designed the study, revised the manuscript and funded the study. All authors have read and agreed to the published version of the manuscript.

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