Original Article

Evaluation of chicken serum and chicken egg yolk as novel supplements for the in vitro cultivation of *Mycoplasma hyorhinis*

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Abstract

Mycoplasma hyorhinis is a significant pathogen in swine, yet its fastidious nutritional requirements present challenges for in vitro cultivation. Conventional supplements like horse serum (HS) often suffer from batch variability and potential inhibitors. This study aimed to evaluate chicken serum (CS) and chicken egg yolk (CY) as novel alternatives to develop a reliable cultivation method for *M. hyorhinis*. Five clinical isolates were cultured for 10 days in a basal medium supplemented with CS or CY at 10%, 20%, or 30%, with 30% HS as the control. Growth was quantified daily using a Color Changing Unit (CCU) assay. CS supported robust growth across strains, with 10% CS outperforming the 30% HS control in most isolates. The 10% CS formulation consistently yielded the highest titers (7.0 Log_{10} CCU/mL) and area under the curve (AUC) values. Notably, increasing CS concentration led to slightly reduced growth. In contrast, CY was less effective, and 30% CY completely inhibited growth. To our knowledge, this is the first comparative study evaluating both chicken serum and egg yolk as alternative supplements for *M. hyorhinis* cultivation, showing that 10% chicken serum is a superior and cost-effective alternative to horse serum, while egg yolk was not supportive under the tested conditions.

Keywords: chicken egg yolk, chicken serum, Color Changing Unit, horse serum, *Mycoplasma hyorhinis*

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Received November 9, 2025
Accepted December 26, 2025
https://doi.org/10.56808/2985-1130.3962

Introduction

Mycoplasma infections are a major concern in the global swine industry. Among the species implicated, *Mycoplasma hyopneumoniae*, *M. hyosynoviae*, and *M. hyorhinis* are responsible for enzootic pneumonia, infectious arthritis, and porcine polyserositis, respectively. These pathogens cause substantial economic losses by impairing growth performance, predisposing pigs to secondary bacterial infections, and aggravating disease severity during co-infections. While *M. hyopneumoniae* has traditionally been the focus of swine respiratory research, *M. hyorhinis* is increasingly recognized as an emerging respiratory pathogen. Recent epidemiological investigations have demonstrated that *M. hyorhinis* is widely prevalent in commercial pig herds worldwide. A cross-sectional study in Central and Eastern Europe further detected *M. hyorhinis* DNA in 100% of sampled herds, confirming its ubiquitous distribution among finishing pigs (Nagy *et al*., 2025). Similarly, a large-scale investigation in mainland China identified *M. hyorhinis* in 31.77% of lung samples collected from pigs showing respiratory symptoms, with several isolates demonstrating high virulence and genetic diversity (Yang *et al*., 2025). Moreover, *M. hyorhinis* has been increasingly detected in tissues beyond the respiratory tract, including the brain, middle ear, and joints (Ko *et al*., 2023). The widespread prevalence and expanding clinical impact of *M. hyorhinis* highlights the urgent need to investigate its pathogenesis and develop effective control strategies. However, a primary bottleneck impeding such research is the inherent difficulty associated with isolating and propagating mycoplasmas *in vitro*.

The fastidious nutritional requirements of mycoplasmas present a significant challenge to their *in vitro* cultivation. These demands are a direct consequence of their minimalist genome. This genomic streamlining results in a limited biosynthetic capacity, evidenced by a restricted proteome and the absence of many core metabolic and enzymatic pathways (Baseman and Tully, 1997; Razin *et al*., 1998). Therefore, successful laboratory propagation is contingent upon the use of complex media, which must be supplemented with animal serum to provide essential lipids, such as fatty acids and cholesterol (Razin, 1969; Slutzky *et al*., 1976).

Although animal serum is a conventional supplement in mycoplasma culture media, its use introduces significant experimental variables and limitations. For instance, horse serum (HS), while widely used, is known for considerable batch-to-batch variation (Friis, 1975), which can range from inconsistent culture performance to direct growth inhibition of specific strains such as *M. pneumoniae* and *M. fermentans* (Sasaki *et al*., 1985). While host-specific options like porcine serum may provide a more physiologically relevant environment for cultivating porcine mycoplasmas such as *M. hyorhinis* and *M. hyopneumoniae*, it presents even greater challenges. Porcine serum exhibits more pronounced batch-to-batch variability than HS, which has been shown to have a marked effect on the growth of key porcine mycoplasmas (Goodwin and Hurrell, 1970). This variability may be attributed to the presence of non-specific inhibitory substances or specific antibodies that can potentially trigger complement-mediated bactericidal activity. Furthermore, the use of homologous serum carries a risk of introducing porcine pathogens and creates interference in downstream analyses. Other sources are generally less effective; bovine serum, for example, is typically suboptimal for porcine mycoplasma species, supporting only limited initial growth (Washburn *et al*., 1978). Ovine serum is particularly unsuitable, exhibiting marked growth inhibition against all porcine mycoplasmas, while rabbit and guinea pig sera also significantly impair colony formation and are not recommended (Roberts, 1971). As an alternative to animal sera, chicken egg yolk (CY) extract has been investigated as a supplement for mycoplasma cultivation, primarily due to its high content of lipoproteins, cholesterol, and phospholipids. Studies have demonstrated that CY-supplemented media exhibit excellent growth-promoting activity for *M. pneumoniae* (Sasaki *et al*., 1983). A particular advantage is that, unlike many batches of HS that exert an inhibitory effect, CY shows no such growth inhibition on *M. pneumoniae* (Sasaki *et al*., 1985). The utility of CY extends to other Mycoplasma species as well; for instance, *M. orale* has been shown to grow better in CY medium than in HS medium, reaching a higher maximum viable cell count more rapidly (Totsuka *et al*., 1985).

While the potential of CY has been explored for human mycoplasmas, the application of CY and avian-derived sera, such as chicken serum (CS), was previously uninvestigated for porcine mycoplasmas at the time this study was initiated. Given the phylogenetic closeness of metabolic pathways between mycoplasma species, we hypothesized that CS might offer a superior lipid profile similar to egg yolk but with fewer inhibitory factors commonly found in horse serum. Consequently, this study aimed to systematically evaluate CS and CY as novel supplements. Specifically, we targeted *M. hyorhinis* due to its growing clinical importance and the persistent challenges in its laboratory isolation. Therefore, this study aimed to systematically evaluate and compare the efficacy of media supplemented with different concentrations of CY and CS as potential substitutes for conventional animal sera in supporting the *in vitro* growth of *M. hyorhinis*.

Materials and Methods

Bacterial strains and verification: The study used five clinical isolates of *M. hyorhinis*, designated A (D110-198), B (D110-232), C (D110-593), D (D110-861), and E (D110-885). These strains were originally isolated in 2021 from swine enzootic pneumonia-like lung lesions of pigs from clinical cases submitted to the Animal Disease Diagnostic Center at National Pingtung University of Science and Technology. For molecular verification, total nucleic acids were extracted from 200 µL of culture using a MagNA Pure 96 Instrument with the MagNA Pure 96 DNA and Viral NA Small Volume Kit (Roche, Mannheim, Germany) according to the manufacturer's protocol, yielding 50 µL of eluate.

Species identity was confirmed using a probe-based quantitative PCR (qPCR) assay on a LightCycler® 96 system (Roche, Basel, Switzerland). The assay targeted a specific region of the *M. hyorhinis* genome using primers Mhr-97F (5'-CCAAGACGATGATGTTTAG CC-3') and Mhr-97R (5'-AAATTCCTTACTGCTGC CTC-3'), along with Universal ProbeLibrary Probe #118 (Roche; 5'-CACTGGGA-3') (Nguyen, 2015). Following verification, all isolates were stored as cryopreserved stocks at -80 °C until use

Preparation of culture media: A serum-free basal medium, modified from the Friis formulation (Friis, 1975), was prepared by combining Friis Broth base (60% v/v), a yeast extract solution (20% v/v; Sigma-Aldrich, St. Louis, MO, USA), and deionized water. After sterilization by autoclaving, the medium was cooled to room temperature, and a sterile, laboratory-prepared 100X supplement solution was aseptically added to a final concentration of 1X. This supplement consisted of D-glucose (50 g/L; Sigma-Aldrich) and L-Arginine HCl (10 g/L; Sigma-Aldrich). A chicken egg yolk solution was prepared based on the method by Sasaki *et al*. (1983). Briefly, Specific-Pathogen-Free chicken egg yolks were aseptically separated, emulsified in 10X phosphate-buffered saline to a 20% (wt/vol) concentration, and clarified by centrifugation at 15,000 ×g for 40 min. Penicillin G was added to the clear supernatant to a final concentration of 500 IU/mL. The prepared CY extract was stored at 4 °C and used within 1 month to ensure stability. From this basal medium, seven distinct experimental media were formulated. Three media were prepared by supplementing the basal medium with chicken serum (Thermo Fisher Scientific, Waltham, MA, USA) to final concentrations of 10%, 20%, and 30% (v/v). Three additional media were prepared by supplementing with CY to final concentrations of 10%, 20%, and 30% (v/v). The final medium, serving as a routine positive control in our laboratory, was prepared by supplementing the aforementioned basal medium with 30% (v/v) horse serum (Cytiva, Marlborough, MA, USA). This single concentration was selected to represent the established cultivation benchmark in our laboratory for comparison purposes. Both CS and HS were used directly without heat inactivation.

Mycoplasma quantification by Color Changing Unit (CCU) assay: The concentration of viable mycoplasmas was quantified using a terminal dilution assay in 96-well microtiter plates. All titrations were performed in triplicate using a proprietary modified Friis medium (kindly provided by Agricultural Technology Research Institute (ATRI), Taiwan; the composition of which is confidential) as the indicator medium. For each sample, ten-fold serial dilutions were prepared. An aliquot of 100 µL from each dilution was transferred to a corresponding well of a 96-well plate pre-filled with 200 µL of fresh indicator medium. A well containing 300 µL of indicator medium alone served as a negative control for each replicate. The plates were incubated aerobically at 37 °C and monitored daily for a color change from red to yellow. The titer (CCU/mL) was calculated as the reciprocal of the highest dilution that exhibited a distinct color change.

Preparation of standardized inoculum: To ensure a consistent starting concentration for all experiments, a standardized inoculum was prepared for each of the five *M. hyorhinis* strains. An initial culture was established by inoculating 300 µL of thawed cryopreserved stock into 3 mL of modified Friis medium and incubating aerobically at 37 °C. The viable cell density of this growing culture was monitored daily using the CCU assay (Section *Mycoplasma quantification by Color Changing Unit (CCU) assay*). Once the culture reached a target concentration of 6.0 Log_{10} CCU/mL, its species identity was re-confirmed by the qPCR assay (Section *Bacterial strains and verification*). This verified and quantified culture was then immediately used as the standardized inoculum.

Growth curve analysis: The growth-promoting efficacy of the seven media was evaluated by conducting a 10-day growth curve analysis for each strain. The experiment was initiated by inoculating 3 mL of each of the seven media formulations with 300 µL of the standardized 6.0 Log_{10} CCU/mL inoculum. All cultures were incubated aerobically at 37 °C for 10 days. To plot the growth curves, an aliquot was aseptically withdrawn from each culture daily, and its viable mycoplasma concentration was immediately determined in triplicate by the CCU assay as described in Section *Mycoplasma quantification by Color Changing Unit (CCU) assay*. To ensure consistency, the same proprietary modified Friis medium was used as the indicator medium for all titrations, regardless of the experimental medium composition.

Statistical analysis: All experiments were performed in three independent biological replicates. Mycoplasma concentrations (CCU/mL) were Log_{10}-transformed prior to analysis. All quantitative results, including endpoint titers (Day 10) and the area under the curve (AUC), are presented as the mean ± standard deviation (SD). The Shapiro-Wilk test was used to assess the normality of the data. As several groups did not follow a normal distribution, statistical comparisons among the seven media formulations were conducted separately for each strain using the non-parametric Kruskal-Wallis test. For pairwise post-hoc comparisons, the uncorrected Dunn's test was selected. Given the limited sample size (n=3) and the frequent occurrence of identical ranks in the dataset, applying conservative corrections for multiple comparisons would disproportionately increase the risk of Type II errors (false negatives). Therefore, the uncorrected test was chosen to preserve statistical power for detecting biologically relevant differences in this exploratory study. All statistical analyses were performed using GraphPad Prism (Version 10.5, GraphPad Software, LLC). A P-value < 0.05 was considered statistically significant

Results

The growth performance of the five *M. hyorhinis* isolates in media supplemented with either horse serum, chicken serum or chicken egg yolk at different concentrations was evaluated based on growth kinetics, final growth yield and AUC. A

comprehensive summary of these results, including final titers and AUC, is presented in Table 1, while the growth kinetics are presented in Figure 1.

Growth kinetics and general observations: To evaluate the efficacy of the seven different media formulations, the growth kinetics of the five *M. hyorhinis* clinical isolates were monitored over a 10-day period. As depicted in the growth curves, all five strains exhibited markedly enhanced proliferation in media supplemented with animal serum (CS and HS) compared to those supplemented with CY (Fig. 1). All serum-supplemented media (CS and HS) supported the growth of all five mycoplasma strains. In these media, the isolates generally entered a logarithmic growth phase within the first 2-3 days and reached a growth plateau by day 5 to 7, with peak titers frequently reaching 7.0 Log_{10} CCU/mL. In contrast, CY-supplemented media demonstrated slower growth rates and limited yield, generally remaining below 5.0 Log_{10} CCU/mL. Notably, supplementation with 30% CY completely inhibited the growth of all isolates throughout the incubation period. The growth profile of Strain A is representative of this trend: titers in all CS and HS media surpassed 6.7 Log_{10} CCU/mL, whereas titers in 10% and 20% CY media remained at or below 5.0 Log_{10} CCU/mL.

Table 1 Comparative summary of growth performance for five *M. hyorhinis* strains in different serum-supplemented media.

Strain	Medium	Final Titer (Log_{10} CCU/mL) (Mean ± SD) (n=3)	Area Under the Curve (Log_{10} CCU/mL × Days) (Mean ± SD) (n=3)
A	CS 10%	7.0 ± 0.0^{a}	54.0 ± 3.5^{a}
	CS 20%	7.0 ± 0.0^{a}	53.7 ± 3.2^{ab}
	CS 30%	7.0 ± 0.0^{a}	51.2 ± 0.6^{ab}
	CY 10%	5.0 ± 0.0^{ac}	37.7 ± 0.6^{bc}
	CY 20%	4.7 ± 0.6^{bc}	33.3 ± 6.4^{c}
	CY 30%	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}
	HS 30%	6.7 ± 0.6^{ab}	45.3 ± 4.9^{ac}
B	CS 10%	7.0 ± 0.0^{a}	55.5 ± 0.0^{a}
	CS 20%	5.0 ± 0.0^{ab}	41.2 ± 0.6^{abc}
	CS 30%	5.0 ± 0.0^{ab}	37.5 ± 0.9^{cd}
	CY 10%	5.0 ± 0.0^{ab}	39.0 ± 0.9^{bd}
	CY 20%	4.7 ± 0.6^{b}	37.0 ± 3.1^{bd}
	CY 30%	0.0 ± 0.0^{b}	0.0 ± 0.0^{d}
	HS 30%	7.0 ± 0.0^{a}	49.7 ± 1.5^{ab}
C	CS 10%	7.0 ± 0.0^{a}	47.7 ± 0.6^{a}
	CS 20%	6.0 ± 0.0^{ab}	41.7 ± 1.2^{ab}
	CS 30%	6.0 ± 0.0^{ab}	39.7 ± 0.6^{abc}
	CY 10%	5.0 ± 0.0^{bc}	31.8 ± 1.2^{bd}
	CY 20%	4.7 ± 0.6^{bc}	27.3 ± 1.9^{cd}
	CY 30%	0.0 ± 0.0^{c}	0.0 ± 0.0^{d}
	HS 30%	5.7 ± 0.6^{ab}	42.7 ± 4.1^{ab}
D	CS 10%	6.3 ± 0.6^{a}	48.8 ± 1.5^{a}
	CS 20%	6.0 ± 0.0^{ab}	44.3 ± 0.6^{a}
	CS 30%	6.0 ± 0.0^{ab}	42.0 ± 0.0^{ab}
	CY 10%	4.7 ± 0.6^{bc}	30.7 ± 4.1^{bc}
	CY 20%	5.0 ± 0.0^{bc}	31.5 ± 2.0^{bc}
	CY 30%	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}
	HS 30%	6.0 ± 0.0^{ab}	40.0 ± 0.0^{ac}
E	CS 10%	7.0 ± 0.0^{a}	51.5 ± 0.0^{a}
	CS 20%	6.3 ± 0.6^{ab}	46.8 ± 2.4^{ab}
	CS 30%	5.0 ± 0.0^{ac}	38.5 ± 0.0^{abc}
	CY 10%	4.7 ± 0.6^{bc}	30.7 ± 4.1^{bd}
	CY 20%	5.0 ± 0.0^{ac}	29.5 ± 1.0^{cd}
	CY 30%	0.0 ± 0.0^{c}	0.0 ± 0.0^{d}
	HS 30%	4.7 ± 0.6^{bc}	29.7 ± 5.0^{bd}

Values represent the mean ± standard deviation (SD) of three independent replicates. Superscript letters (e.g., a, b, c) within the same column for each strain indicate statistically significant differences between groups ($P < 0.05$); groups sharing a common letter are not significantly different.

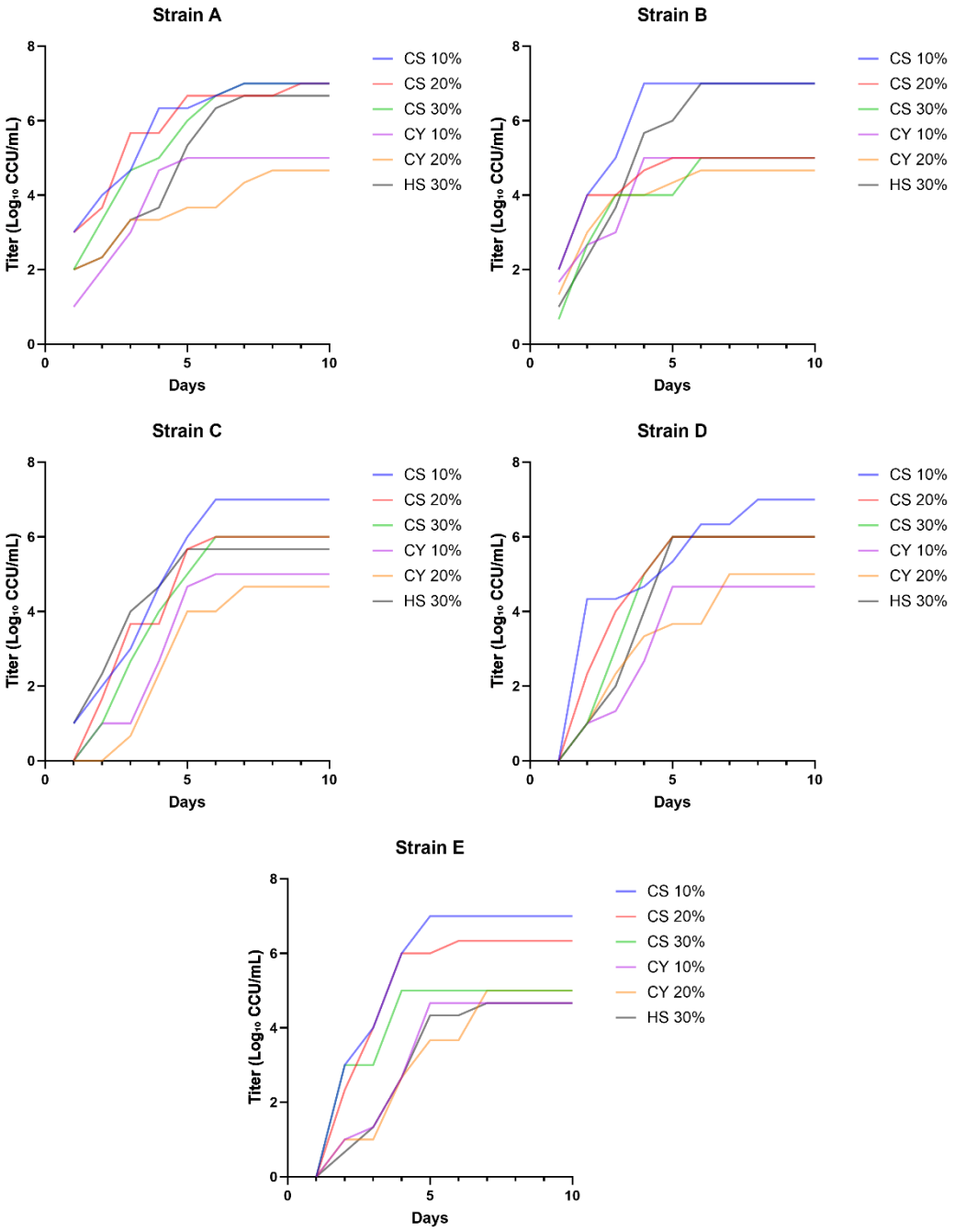

Figure 1 Growth curves of five *M. hyorhinis* isolates in media supplemented with different sera. The growth curves of five clinical isolates were determined after 10 days of incubation in modified Friis medium supplemented with 10%, 20%, or 30% of either chicken serum (CS) or chicken egg yolk (CY) or 30% horse serum (HS). The five panels show the growth kinetics for Strain A, B, C, D and E.

Final growth yield: The final growth yield (day 10) varied significantly among the different media formulations (Table 1, Fig. 2). Final titers ranged from no detectable growth to a maximum of 7.0 Log_{10} CCU/mL. Notably, the maximum titers were most consistently achieved in media supplemented with 10% CS and also by Strain B in 30% HS. Conversely, the lowest non-zero titers (approximately 4.7 Log_{10} CCU/mL) were recorded primarily in CY-supplemented media, although Strain E exhibited a similarly low titer in 30% HS. An inverse concentration-dependent relationship was observed for CS media; for four of the five strains, increasing the CS concentration resulted in a slight to moderate decrease in the final titer. For instance, the titer for Strain E decreased from 7.0 ± 0.0 Log_{10} CCU/mL in 10% CS to 5.0 ± 0.0 Log_{10} CCU/mL in 30% CS. Only Strain A maintained a uniformly high titer of 7.0 Log_{10} CCU/mL across all CS concentrations.

Statistical analysis confirmed that serum-based media consistently outperformed CY-based media (Fig. 2). Specifically, the 10% CS medium yielded significantly higher titers compared to various CY concentrations for Strains B, C, D, and E ($P < 0.05$). Strain A exhibited robust growth across all CS concentrations, which were statistically superior to the 20% and 30% CY groups.

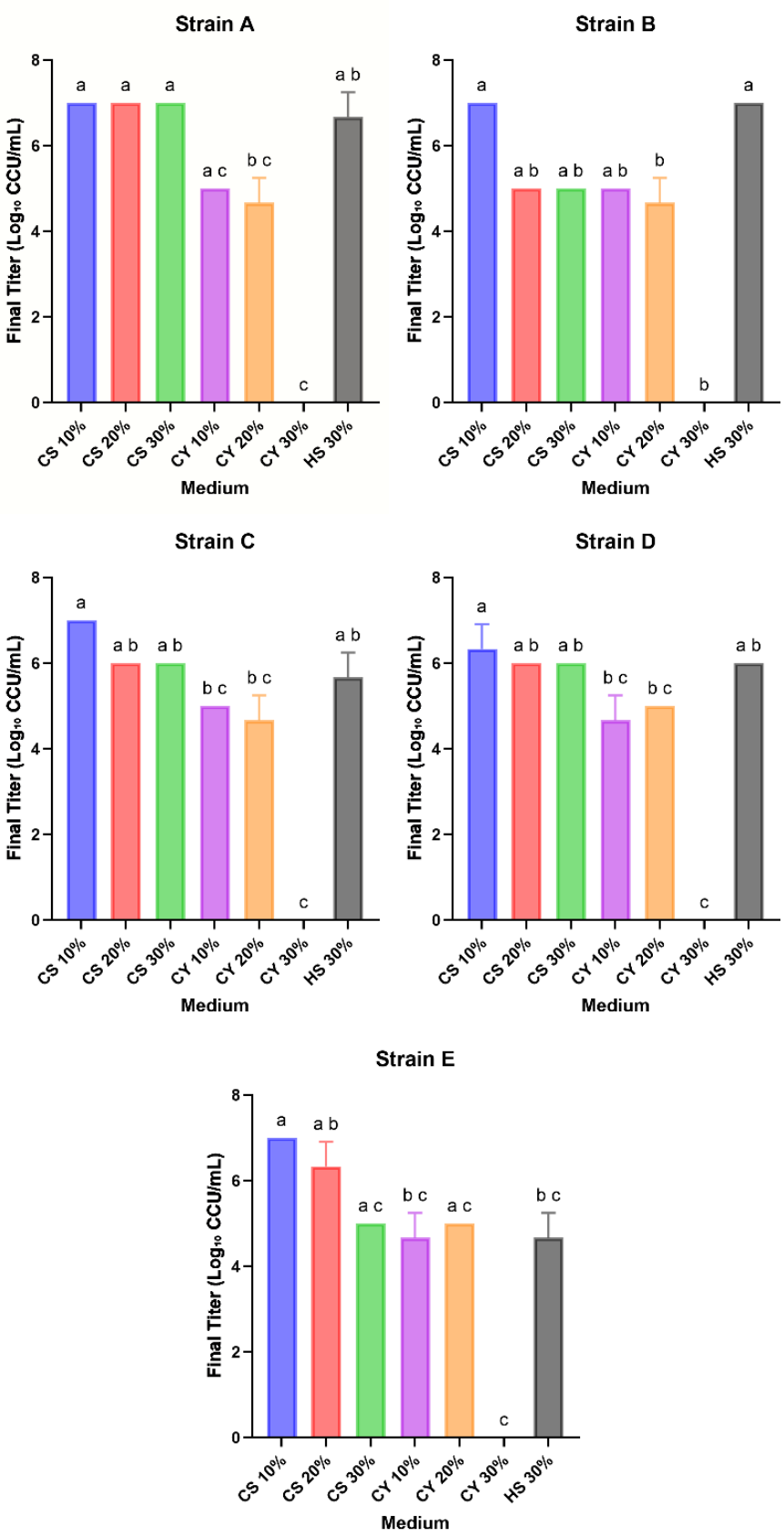

Figure 2 Final growth yield of five *M. hyorhinis* isolates in media supplemented with different sera. The final titers of five clinical isolates were determined after 10 days of incubation in modified Friis medium supplemented with 10%, 20%, or 30% of either chicken serum (CS) or chicken egg yolk (CY) or 30% horse serum (HS). The five panels show the results for Strain A, B, C, D and E. Bars indicate the mean final titer (Log_{10} CCU/mL) of three independent biological replicates (n=3), with error bars representing the standard deviation (SD). Different lowercase letters (a, b, c) above the bars indicate a statistically significant difference between groups ($P < 0.05$); groups not sharing a common letter are significantly different.

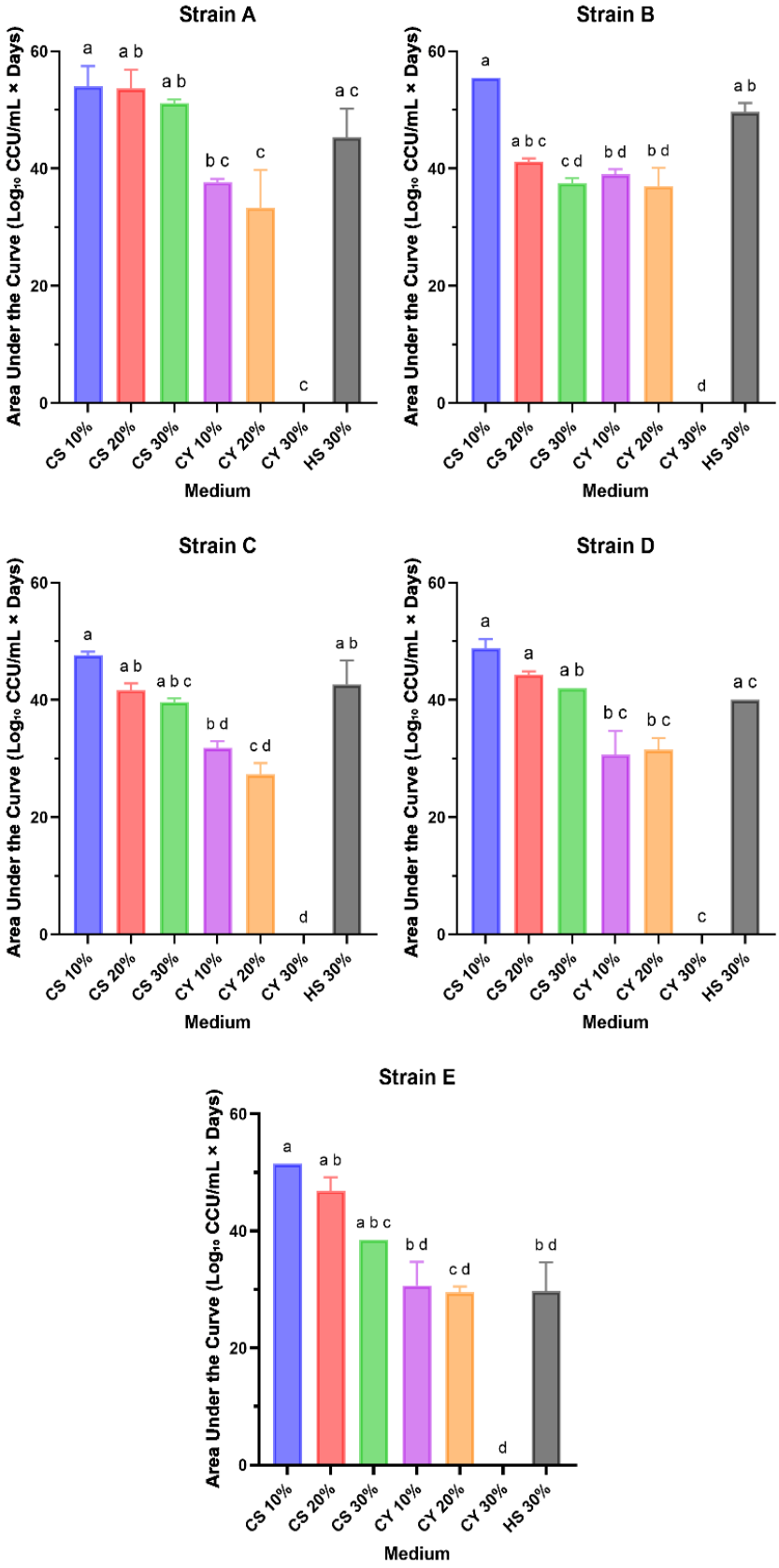

Figure 3 Area under the curve (AUC) of five *M. hyorhinis* isolates in media supplemented with different sera. The AUC values of five clinical isolates were determined after 10 days of incubation in modified Friis medium supplemented with 10%, 20%, or 30% of either chicken serum (CS) or chicken egg yolk (CY) or 30% horse serum (HS). The five panels show the results for Strain A, B, C, D and E. Bars indicate the mean AUC (Log_{10} CCU/mL × Days) of three independent biological replicates (n=3), with error bars representing the standard deviation (SD). Different lowercase letters (a, b, c) above the bars indicate a statistically significant difference between groups ($P < 0.05$); groups not sharing a common letter are significantly different.

Area under the curve: To provide a comprehensive assessment of overall growth, the AUC was calculated for each isolate under all media conditions (Table 1, Fig. 3). Serum-supplemented media generally yielded significantly higher AUC values than CY-supplemented media ($P < 0.05$). An inverse concentration-dependent relationship was also observed with CS supplementation; increasing the CS concentration from 10% to 30% led to a progressive decrease in the AUC for all strains. Due to no growth, the 30% CY medium resulted in an AUC of zero for all isolates. The 10% CS formulation was frequently the most effective medium overall. Statistically, the AUC for 10% CS was significantly higher than that of any CY-supplemented medium for all five strains ($P < 0.05$). The performance of 30% HS was generally comparable to that of the high-performing CS groups. However, a notable strain-specific exception was observed with Strain E, where the 30% HS medium produced a significantly lower AUC (29.7 ± 5.0 Log_{10} CCU/mL × Days) than the 10% CS medium (51.5 ± 0.0 Log_{10} CCU/mL × Days), with its performance being statistically indistinct from the suboptimal CY media.

Discussion

This study reveals that supplementation of a modified Friis medium with 10% chicken serum facilitates high-titer growth of clinical *M. hyorhinis* isolates, with growth yields frequently surpassing those achieved with the 30% horse serum control. This formulation supported growth to the maximum measured titer of 7.0 Log_{10} CCU/mL for four of the five strains tested. Moreover, our results highlight two phenomena with significant practical implications: an inverse concentration-dependent relationship for CS, where concentrations above

The superior performance of the 10% CS medium over the conventional 30% HS formulation may be attributed to key differences in lipid composition and the presence of growth-inhibitory factors. Mycoplasmas exhibit an absolute requirement for cholesterol for their membrane synthesis. Previous research demonstrated that CY supported the growth of *M. pneumoniae* more effectively than HS, a phenomenon partly associated with CY's substantially higher concentration of free cholesterol and phospholipids, which is approximately eight times that of HS (Sasaki *et al*., 1983). Given that mycoplasmas preferentially incorporate unesterified cholesterol for membrane synthesis (Sasaki *et al*., 1983; Sasaki and Kihara, 1987). Similarly, CS likely possesses a favorable lipid profile, supported by evidence that the very low-density lipoprotein (VLDL) fraction is identical in chicken serum and egg yolk, with high-density lipoproteins (HDL) also showing marked similarities (Hillyard *et al*., 1972). Therefore, the lipoprotein structure in CS may offer a readily bioavailable source of essential lipids, facilitating more efficient cholesterol uptake and vigorous proliferation for *M. hyorhinis*. A second advantage of CS may be a lower concentration of inherent growth inhibitors. It is well-documented that many batches of HS, even after heat inactivation (Sasaki *et al*., 1985). Thus, the superior efficacy of 10% CS suggests it contains a lower burden of these inhibitory compounds, creating a more permissive growth environment. This may reflect either an intrinsically lower level of inhibitory compounds in chicken serum versus horse serum, or a concentration-dependent effect where the 10% CS medium simply contains fewer total inhibitors than the 30% HS control. Either scenario would result in a more stable and permissive growth environment for *M. hyorhinis*.

A key finding of this study was the inverse concentration-dependent effect observed with CS supplementation; a 10% concentration consistently provided optimal growth, whereas higher concentrations of 20% and 30% resulted in a modest but discernible decrease in both final titers and overall growth as measured by AUC. While some literature suggests that higher serum levels generally enhance growth (Taylor-Robinson and Behnke, 1987), our observation is consistent with studies that report growth inhibition at high serum concentrations for certain strains (Roberts, 1971; Del Giudice *et al*., 1980). We hypothesize that the potential explanation involves lipid-mediated cytotoxicity. While mycoplasmas require lipids, an excess of certain components, such as free fatty acids (FFAs), can be toxic and lead to cell lysis (Rodwell, 1969). Although serum albumin buffers fatty acids, an imbalance between supply and binding capacity can lead to the accumulation of toxic unbound FFAs (Rodwell, 1969; Cluss and Somerson, 1986). Alternatively, this effect may be related to the concentration of latent growth-inhibitory substances. As previously discussed, animal sera can contain various proteinaceous inhibitors. While 10% CS formula appears to have a lower inhibitory load than 30% HS medium, increasing the CS concentration would proportionally increase the total amount of any such latent factors in the medium. This elevated concentration of inhibitors could be sufficient to partially counteract the benefits of increased nutrient availability, resulting in the observed inverse concentration-dependent outcome. Beyond biochemical factors, physicochemical properties such as increased osmolarity or viscosity at higher serum concentrations (20–30%) could potentially impede nutrient transport or alter the microenvironment, although this remains to be verified. Taken together, the 10% CS formulation likely represents an optimal equilibrium, providing sufficient concentrations of essential nutrients while maintaining potential inhibitory or cytotoxic factors below a threshold that would impair cell growth.

In contrast to the success of CS, and despite being rich in key lipids required for mycoplasma growth, CY medium proved unsuitable for the in vitro cultivation of *M. hyorhinis*, with complete growth inhibition observed at a 30% concentration. This finding is particularly noteworthy given that CY has previously been reported to outperform HS for the cultivation of other species like *M. pneumoniae* (Sasaki *et al*., 1985), but such species-dependent responses to CY have been previously documented. For instance, studies on oral mycoplasmas revealed that while *M. orale* grew well in a CY-based medium, the growth of *M. salivarium* was less supported, and *Ureaplasma urealyticum* failed to grow entirely (Totsuka *et al*., 1985). The failure of CY in supporting *M. hyorhinis* growth may be multifactorial,

with one primary reason likely being the lack of sufficient lipid carriers. Egg yolk extract contains a significantly lower total protein concentration than animal serum, approximately one-fourth that of HS (Sasaki *et al*., 1983). Mycoplasma cultivation requires fatty acids to be delivered in a utilizable and non-toxic form, a role typically fulfilled by serum albumin (Razin *et al*., 1998). The relative deficiency of such carrier proteins in CY may have led to an inability to buffer the cytotoxic effects of high concentrations of free fatty acids, resulting in growth inhibition or cell lysis (Rodwell, 1969). Furthermore, the physicochemical instability of CY lipoproteins is a well-documented issue. Egg yolk lipoproteins are prone to denaturation and the formation of insoluble debris, particularly after freezing or during prolonged storage (Sasaki *et al*., 1983; Totsuka *et al*., 1985). Additionally, CY-supplemented media are known to exhibit increased turbidity and precipitation, potentially through interactions between lipoproteins and other media components (Sasaki *et al*., 1985). Although this study utilized a liquid medium, it is conceivable that at high concentrations, the unrefined CY extract formed inhibitory aggregates due to these unstable lipid structures, thereby impeding the growth of *M. hyorhinis*. Collectively, the combination of potential lipotoxicity due to insufficient protein carriers and physicochemical instability likely rendered the CY supplement unsuitable for the cultivation of this particular mycoplasma species.

Beyond the biological evaluation, assessing the cost-efficiency of these supplements is crucial for practical implementation. An analysis of cost-efficiency reveals that the 10% CS formulation is not only effective in promoting growth but also the most economical choice (Table 2). It achieved a higher average maximum titer (6.8 Log_{10} CCU/mL) at a substantially lower approximate cost (387 NTD/100mL) compared to the conventional 30% HS medium (6.2 Log_{10} CCU/mL at 600 NTD/100mL). This highlights that a superior biological outcome can be achieved with reduced expenditure. Furthermore, the inverse concentration-dependent effect of CS is reinforced from a financial perspective, doubling or tripling the concentration to 20% or 30% dramatically increased material costs without a corresponding improvement in cultivation yield. While egg yolk extract is considerably less expensive, its poor growth-supporting ability renders it an inefficient and impractical option. Therefore, considering both cultivation efficacy and cost, the 10% CS formulation emerges as the optimal supplement for the in vitro propagation of *M. hyorhinis*.

While this study provides strong evidence for the efficacy of chicken serum, certain limitations should be acknowledged. The findings are based on five clinical isolates from a single diagnostic center; further validation using a larger, diverse collection would be beneficial. Additionally, this study evaluated growth kinetics solely in liquid broth systems; the efficacy of CS for colony formation on solid agar plates or for primary isolation directly from clinical specimens remains to be validated. Moreover, our discussion regarding the underlying mechanisms, such as favorable lipid profiles and a lower concentration of inhibitors in CS, is inferential and based on existing literature rather than direct compositional analysis of the specific supplements used. Future research employing proteomic and lipidomic analyses could definitively identify and quantify the specific factors within chicken and horse serum that contribute to the observed differences in growth promotion. Such studies would provide a more complete mechanistic understanding and further refine mycoplasma culture methodologies.

Table 2 Cost-effectiveness of chicken serum, egg yolk, and horse serum for *M. hyorhinis* cultivation.

Medium	Cost (NTD/100mL)[1]	Max CCU (Avg.)[2]
HS 30%	600	6.2
CS 10%	387	6.8
CS 20%	774	6.4
CS 30%	1160	6.2
CY 10%	60	5
CY 20%	90	5

[1]Costs are presented as approximate values in New Taiwan Dollars (NTD) per 100 mL of the final medium. These values are based on the market prices of the supplements at the time the study was conducted.
[2]Max CCU (Avg.) represents the mean maximum titer (Log_{10} CCU/mL) calculated across all five strains tested.

In conclusion, this study is the first, to our knowledge, to demonstrate that chicken serum is a highly effective growth supplement for mycoplasma cultivation. We have established that a 10% CS formulation serves as a superior and more cost-effective alternative to the conventional 30% HS medium for clinical *M. hyorhinis* isolates. In contrast to chicken egg yolk, which was found to be unsuitable, 10% CS provides an optimized methodology that can enhance the consistency and reduce the expense of cultivating this fastidious pathogen, representing a significant practical advancement for both diagnostic and research applications.

Acknowledgments

We would like to express our sincere gratitude to Dr. Chun-Hung Lin for his invaluable guidance, and to both him and the Agricultural Technology Research Institute, Taiwan, for providing the proprietary enriched medium for this study. We also thank all colleagues and students of the Immunology Laboratory and the Veterinary Pathology Laboratory at National Pingtung University of Science and Technology for their assistance in the preliminary collection of bacterial strains.

Declaration of generative AI and AI-assisted technologies in the writing process: During the preparation of this manuscript, the authors used Gemini 2.5 Pro (Google) for the purposes of superficial text editing (e.g., grammar, spelling, punctuation, and formatting). The authors have reviewed and edited the output and take full responsibility for the content of this publication.

References

Baseman JB and Tully JG 1997. Mycoplasmas: Sophisticated, reemerging, and burdened by their notoriety. Emerg Infect Dis. 3: 21–32.

Cluss RG and Somerson NL 1986. Interaction of albumin and phospholipid:cholesterol liposomes in growth of *Mycoplasma* spp. Appl Environ Microbiol. 51: 281–287.

Del Giudice RA, Gardella RS and Hopps HE 1980. Cultivation of formerly noncultivable strains of *Mycoplasma hyorhinis*. Curr Microbiol. 4: 75–80.

Friis NF 1975. Some recommendations concerning primary isolation of *Mycoplasma suipneumoniae* and *Mycoplasma flocculare* a survey. Nord Vet Med. 27: 337–339.

Goodwin RFW and Hurrell JMW 1970. Further observations on the problem of isolating *Mycoplasma suipneutnoniae* from field cases of enzootic pneumonia in pigs. J Hyg. 68: 313–325.

Hillyard LA, White HM and Pangburn SA 1972. Characterization of apolipoproteins in chicken serum and egg yolk. Biochemistry 11: 511–518.

Ko CC, Merodio MM, Spronk E, Lehman JR, Shen H, Li G, Derscheid RJ and Piñeyro PE 2023. Diagnostic investigation of *Mycoplasma hyorhinis* as a potential pathogen associated with neurological clinical signs and central nervous system lesions in pigs. Microb Pathog. 180: 106172.

Nagy EZ, Földi D, Madzig F, Wehmann E, Orosz A, Kempf A, Buza L, Mátyus J, Búza L, Grózner D, Kreizinger Z and Gyuranecz M 2025. Cross-sectional study of *Mycoplasma hyopharyngis*, *Mycoplasma hyopneumoniae*, *Mycoplasma hyorhinis* and *Mycoplasma hyosynoviae* in the tonsils of fattening pigs from Central-Eastern Europe. Porc Health Manag. 11: 11.

Nguyen XT 2015. Detection of *Mycoplasma hyopneumoniae, Mycoplasma hyorhinis,* PCV2 and PRRSV in Taiwanese Suckling Pigs (Master Thesis). Pingtung: National Pingtung University of Science and Technology. 78 pp.

Razin S 1969. Structure and function in mycoplasma. Annu Rev Microbiol. 23: 317–356.

Razin S, Yogev D and Naot Y 1998. Molecular biology and pathogenicity of mycoplasmas. Microbiol Mol Biol Rev. 62: 1094–1156.

Roberts DH 1971. Interaction of porcine mycoplasmas with fresh animal serum. J Hyg. 69: 361–368.

Rodwell AW 1969. The supply of cholesterol and fatty acids for the growth of mycoplasmas. J Gen Microbiol. 58: 29–37.

Sasaki T and Kihara K 1987. Utility of egg yolk medium for cultivation of some mycoplasma species. Microbiol Immunol. 31: 491–496.

Sasaki T, Shintani M and Kihara K 1983. Utility of egg yolk medium for cultivation of *Mycoplasma pneumoniae*. J Clin Microbiol. 18: 1167–1173.

Sasaki T, Shintani M and Kihara K 1985. Comparison of the efficacy of egg yolk extract and horse serum for growth of *Mycoplasma pneumoniae*. Microbiol Immunol. 29: 499–507.

Slutzky GM, Razin S, Kahane I and Eisenberg S 1976. Serum lipoproteins as cholesterol donors to mycoplasma membranes. Biochem Biophys Res Commun. 68: 529–536.

Taylor-Robinson D and Behnke J 1987. The prolonged persistence of mycoplasmas in culture. J Med Microbiol. 23: 89–92.

Totsuka M, Hayashi H, Shibata K and Watanabe T 1985. The growth of oral mycoplasmas in chicken egg yolk medium. Jpn J Oral Biol. 27: 423–429.

Washburn LR, Hughes JH and Somerson NL 1978. Mycoplasma growth factors in bovine serum fraction. J Bacteriol. 135: 818–827.

Yang F, Yang L, Duan X, Qian Y, Ma H, Jia X, Huo X, Dong W, Chen H and Tan C 2025. Isolation and identification of *Mycoplasma hyorhinis* and virulence evaluation of its field isolates. Front Vet Sci. 12: 1542992.