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ORIGINAL ARTICLE

Optimization of the composition of a solid culture medium for *Mycobacterium avium* subsp. *paratuberculosis* using factorial design and response surface methodology

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Abstract

Aim: To develop an optimized solid culture medium for improved growth of *Mycobacterium avium* subsp. *paratuberculosis* (MAP).

Methods and results: Seven medium constituents (factors) were assessed at various concentrations for their ability to positively affect MAP growth. The factors tested were Tween 80, egg yolk, casitone, taurocholic acid, Mycobactin J, agar and either OADC or ADC supplement. After an initial screening of individual factors, a fractional factorial design and a response surface methodology (RSM) central composite design were used to assess the effects of multiple factors simultaneously and design a new solid culture medium. MAP growth became visible on streak plates of the optimized solid medium 2 weeks earlier than on Herrold's egg yolk medium (HEYM).

Conclusions: MAP grew faster on the optimized solid medium than on HEYM. It consisted of Middlebrook 7H9 broth with 1.0% Tween 80, 0.019% casitone, 1.4% bacteriological agar, 10% egg yolk, 10% ADC and 1.65 μg ml^{-1} Mycobactin J.

Significance and impact of the study: This is the first study to use an RSM approach to optimize the composition of a solid medium for MAP culture. The new medium could improve MAP culture in future by reducing incubation times and increasing MAP colony numbers.

KEYWORDS

egg yolk, growth supplements, medium composition optimization, *Mycobacterium avium* subsp. *paratuberculosis*, response surface methodology, solid culture

INTRODUCTION

Mycobacterium avium subsp. *paratuberculosis* (MAP) is the causative agent of Johne's disease (also known as Paratuberculosis), a chronic wasting disease that primarily affects ruminants (Imada et al., 2020). It is also a suspected zoonotic pathogen with possible links to several human autoimmune disorders, most notably Crohn's disease (Waddell et al., 2015). The role of MAP in the development of these conditions is uncertain, but control of human exposure via milk and dairy products principally has previously been recommended as a precaution (Advisory Committee on the Microbiological Safety of Food, 2002). Several countries have implemented control programs to reduce the spread of MAP infection in livestock used for food production (Donat et al., 2020), but these are hampered

by a lack of sensitive and specific MAP detection methods (Szteyn et al., 2020). Immunological methods (such as blood or milk ELISA) lack sensitivity, particularly in the early stages of the disease (McAloon et al., 2020), and methods based on the detection of genetic material (such as PCR) are typically more sensitive but cannot differentiate between live and dead MAP cells (Plain et al., 2020). In order to demonstrate the viability of MAP detected by PCR, this method has been combined with viability dyes such as propidium monoazide (Cechova et al., 2021; Kralik et al., 2010), or bacteriophages as MAP lysing agents, since these only infect live bacteria (Foddai & Grant, 2020; Swift et al., 2020). Research continues to address the limitations of existing detection methods for MAP.

Culture is considered the 'gold standard' method for the detection of viable MAP, against which the sensitivity and specificity of novel detection methods are commonly measured (Whittington, 2020). Advantages of culture include confirmation of the presence of viable MAP and facilitation of functional studies on the isolated strains. However, current culture methods have severe limitations, as MAP is extremely slow growing with a generation time of over 20 h (Rowe & Grant, 2006) and primary isolation typically taking 8–20 weeks, with some studies reporting isolation only after incubation for several months (Whittington, 2020). Therefore, new innovations in relation to cultural approaches are required to achieve an improved culture of MAP.

Most recent studies reporting developments in MAP culture methods have focused on the culture of MAP in liquid media (Pozzato et al., 2011; Whittington et al., 2013). This is because MAP grows more quickly in broth compared to agar, with primary isolation of MAP typically taking 8–12 weeks in broth compared with 12–20 weeks on a solid medium (Whittington, 2020). Despite this, culture on solid media has many advantages over broth culture as isolated colonies will facilitate: identification of suspected MAP by typical colony morphology, enumeration of load, MAP can potentially be distinguished from other bacteria in mixed cultures and colonies may be stored for future study or molecular typing. Therefore, the development of an improved solid culture medium for a more efficient culture of MAP would be of benefit to the field of Paratuberculosis diagnosis and research.

Traditionally, novel culture media are developed by testing the effects of various concentrations of supplements on bacterial growth one supplement at a time, and the optimal concentration of each supplement is selected before moving on to study the next supplement. However, this approach is slow and laborious, particularly, when studying a slow-growing bacterium such as MAP. It also does not take account of potential synergistic or antagonistic interactions between supplements. An alternative approach is to use response surface methodology (RSM). This is a statistical approach used to determine the optimum conditions for a multivariable system using the minimum number of experiments (He et al., 2004). With an RSM approach, the effects of several supplements may be investigated in one experiment, improving the efficiency of culture media optimization. He et al. (2004) and Chaliha et al. (2020) both demonstrated the advantages of using an RSM approach when they used a central composite design (CCD) to optimize fermentation conditions for enhanced growth of *Clostridium butyricum* and to optimize three different culture media for isolation of the fastidious fungus *Exobasidium vexans*, respectively.

Several media supplements, Mycobactin J, egg yolk, Polysorbate 80 (Tween 80), taurocholic acid, casitone, bacteriological agar and a pairwise comparison of ADC and OADC, were selected to investigate their effects on MAP culture. Mycobactin J, egg yolk, casitone and Tween 80 have all been used for MAP culture previously but were added to growth media at different concentrations by different studies, and so they were included with the aim of determining the optimum concentration of each in a novel growth medium. ADC and OADC are both used to supplement Middlebrook media for MAP culture, but these supplements have not recently been compared to determine which is superior. Taurocholic acid has not been used for MAP culture previously, but it was included as the growth rate of some bacteria that survive in the intestines (e.g. *Lactobacillus brevis* KB290, Kimoto-Nira et al., 2015) may be increased by the presence of bile acids in the growth medium. Finally, concentrations of bacteriological agar below the usual 1.5% w/v were utilized in this study. The rationale was that MAP growth rates have been observed to be higher in broth compared with agar, which may reflect the fact that MAP cells growing in broth have greater mobility and consequently have greater access to nutrients and less exposure to toxic waste products (Bonnet et al., 2020). By reducing the agar concentration, the latter might be improved for MAP cells growing on a solid medium also.

In this study, our aim was to use computational methods and multivariate modelling to develop an optimized solid culture medium for the more rapid culture of MAP. For this purpose, several factors (as outlined above) were selected for investigation. To test these factors efficiently, a stepwise approach was adopted that started with factorial modelling and led to RSM CCD.

MATERIALS AND METHODS

Bacterial cultures

All MAP stock cultures were freshly prepared from cryobead (Technical Service Consultants Ltd.) stocks stored at

−80°C. For each culture, 10 ml 7H9/OADC/Mycobactin J broth was inoculated with one cryobead. The cultures were incubated at 37°C for approximately 4 weeks. Prior to use, MAP cultures were de-clumped by sonication on ice in an UltraSonic PH 30 sonicator bath (Fisher Scientific Ltd.) at 37 Hz and 100 V for 4 min in Pulse mode (Foddai & Grant, 2015). The OD_{600nm} was then standardized to 0.1 (approximately 10^7 cfu MAP ml^{-1}), before a 10-fold dilution series to 10^{-5} was prepared in phosphate-buffered saline (PBS, Fisher Scientific). For the screening experiment, agar slopes were inoculated with 100 µl of the 10^{-4} dilution of MAP ATCC 19698, while 25 mL flasks were inoculated with 1 ml of the 10^{-5} dilution (anticipating 100–200 colonies in both cases). For the fractional factorial experiment and the RSM experiment, Petri plates were inoculated with 200 µl of the 10^{-4} MAP 796 PSS dilution (anticipating 200–300 MAP colonies) per plate. For the final experiment comparing the growth of multiple strains of MAP on the new medium, an additional two MAP strains from the QUB culture collection were used (MAP B4, a bovine isolate and MAP VIAS-Crohn's, an isolate from a Crohn's patient). All four strains were MAP C-strains since no MAP S-strains were available within the laboratory culture collection to test. All cultures were incubated at 37°C until growth appeared or more than 4 weeks had passed.

Media preparation

Middlebrook 7H9 broth (Becton, Dickinson and Co.) was used as the base for all culture media formulations. Bacteriological agar (Oxoid) was added prior to autoclaving at 121°C for 10 min, other supplements after autoclaving and cooling of the base medium to 55°C. The 7H9/OADC/Mycobactin J broth used for culture of MAP stock solutions was supplemented with 10% v/v OADC (HiMedia Laboratories) and 2 µg ml^{-1} Mycobactin J (Synbiotics Europe SAS).

For the screening experiment, 7H9 medium was supplemented with 15 g agar, 100 ml ADC, OADC (both HiMedia Laboratories), or sterile water, and 2 mg Mycobactin J/L. A sterile stock solution of Tween 80 (Sigma-Aldrich) was prepared by autoclaving, while a 100 mg ml^{-1} taurocholic acid solution was prepared by dissolving sodium taurocholate (Sigma-Aldrich) in distilled water and filter-sterilizing by passage through a 0.45 µm PVDF syringe filter (Millex-HV; Merck KGaA). Each of these supplements was added to the final concentrations given below directly prior to pouring. Media supplemented with Tween 80 was set in 10 ml agar slopes in 25 ml universal tubes (Fisher Scientific) instead of Petri dishes to reduce desiccation of the media, while media supplemented with taurocholic acid was added in 6 ml aliquots to 25 ml sterile, nontreated plug seal tissue culture flasks (VWR International Ltd). All media formulations in the screening experiment were tested in triplicate, while for all other experiments, they were tested in duplicate.

For the fractional factorial and RSM experiments, stock solutions of the various intended supplements were prepared. Tween 80 and a 450 mg ml^{-1} taurocholic acid solution were prepared as previously described. Mycobactin J was prepared by resuspending 2 mg in 4 ml of 100% ethanol (Sigma-Aldrich) to create a 500 µg ml^{-1} solution. Casitone (Becton, Dickinson and Co.) was dissolved in distilled water to create a 10% w/v solution, which was autoclaved. The egg yolk was prepared from fresh eggs purchased from a local supermarket, by aseptically separating the yolks into a sterile flask and then mixing until homogenous. ADC and OADC were used as purchased from HiMedia Ltd, Germany. The concentrations of each supplement per medium formulation (run) are available in Tables S1 and S2. Media was set in 10 ml amounts in 60 mm Petri dishes.

Herrold's egg yolk medium (HEYM) was made in-house using 9 g peptone (Becton, Dickinson and Co.), 4.5 g sodium chloride (VWR International Ltd), 15 g bacteriological agar, 2.7 g beef extract (Oxoid), 4.1 g sodium pyruvate (Sigma-Aldrich) and 27 ml glycerol per 900 mL of water. The medium was adjusted to pH 7.8 and autoclaved at 121°C for 15 min prior to the addition of 100 ml fresh egg yolk, 5 ml 2% w/v malachite green (Sigma-Aldrich), and 2 mg Mycobactin J to the cooled (55°C) medium. HEYM, 7H9/OADC/Mycobactin J agar and the new optimized medium were set in 20 mL amounts in 90 mm Petri dishes. Petri dishes from all experiments were wrapped in a double layer of laboratory sealing film (Diversified Biotech) after inoculation to prevent desiccation during incubation.

Screening experiments and selection of factors for RSM

The effect of five different concentrations of taurocholic acid (0, 0.5, 5, 15 and 25 mg ml^{-1}), added to 7H9/OADC/Mycobactin J agar, and Tween 80 (0, 0.001, 0.01., 0.1 and 1.0% v/v), added to 7H9/Mycobactin J agar with and without 10% OADC or ADC added, on MAP growth was assessed during the screening stage. The cultures were examined periodically up to 60–70 days of incubation at 37°C, and the number of MAP colonies was assessed qualitatively on each occasion (no visible growth, <100 colonies or ≥100 colonies).

Fractional factorial design experiment

Initially, six supplements ('factors') were selected to test their effect on MAP growth: Tween 80, taurocholic acid, Mycobactin J, bacteriological agar, casitone and egg yolk. The base medium in all cases was 7H9 broth with 10% (v/v) ADC added. To efficiently investigate the effects of these factors on MAP culture, a half-fraction two-level factorial design with 32 runs was implemented, where each numerical factor is varied over two levels. In addition, three central points were manually added to improve accuracy. Design Expert 13 software (Stat-Ease, Inc.) was used to calculate the final Design of Experiment (DoE), which consisted of 35 agar formulations (runs), the composition of which are given in Table S1. Two separate responses were measured: $R1$ – no. of days until first colonies became visible to the naked eye and $R2$ – no. of colonies per run on day 37. At the conclusion of the fractional factorial experiment, colonies from all suspected positive runs were confirmed by acid-fast staining using a Quick-TB acid-fast staining kit (RAL Diagnostics SAS, Martillac, France). The results were used to select three to four key factors to further investigate with RSM.

RSM/CCD experiment

Based on the results of the fractional factorial design experiment, three factors were selected (Tween 80, casitone and egg yolk) for further analysis using RSM with a Face-Centred CCD with three levels of each supplement (factor) selected on the basis of the Fractional factorial experiment. The results were fitted to a polynomial regression model to predict the optimal combination of factors. The equation is:

$$Y = \beta_0 + \sum_i \beta_i X_i + \sum_i \beta_{ii} X_i^2 + \sum_{ij} \beta_{ij} X_i X_j,$$

where Y is the response, β_0 is the constant coefficient, β_i is the linear coefficient, β_{ii} the quadratic coefficient, β_{ij} is the second-order interaction coefficient, and X_i and X_j are independent variables.

All design parameters were submitted to Design Expert 13 software to calculate the DoE, which consisted of a total of 20 runs. These were duplicated to compare the effects when supplementing media with ADC versus OADC (both added at a concentration of 10% v/v), resulting in 40 runs altogether (see Table S2 for agar compositions). The comparison of ADC versus OADC was a categorial value in the RSM design. The same amounts of Mycobactin J (1.65 μg ml^{-1}) and bacteriological agar (1.4% w/v) were added to all agars (runs).

For the RSM experiment, three responses were measured: $R1$ – no. of days until first colonies became visible to the naked eye, $R2$ – no. of colonies per run on day 33 and $R3$ – no. of days until maximum numbers were reached (i.e. colony numbers levelled off and 'entered stationary phase'). Again, the average of two replicates was used unless one replicate was unmeasurable due to contamination. At the conclusion of the experiment, colonies from a small number of runs were selected for acid-fast staining using the Quick-TB acid-fast staining kit. Colonies from the other runs were identified by comparing colony morphology to colonies previously confirmed to be MAP either in this experiment or the fractional factorial experiment.

Finally, the growth of four strains of MAP (ATCC 19698, B4, 796 PSS and VIAS-Crohn's from the QUB culture collection) on the optimized medium indicated by the results of the RSM experiment was compared to MAP growth on HEYM and 7H9/OADC/Mycobactin J agars by streaking a 10 μl inoculum across the agar and incubating until colonies became visible.

Statistical analysis and model validation

During the fractional factorial and RSM experiments, the number of colonies per agar per day was recorded in GraphPad Prism (Figures 1 and 3). These results were

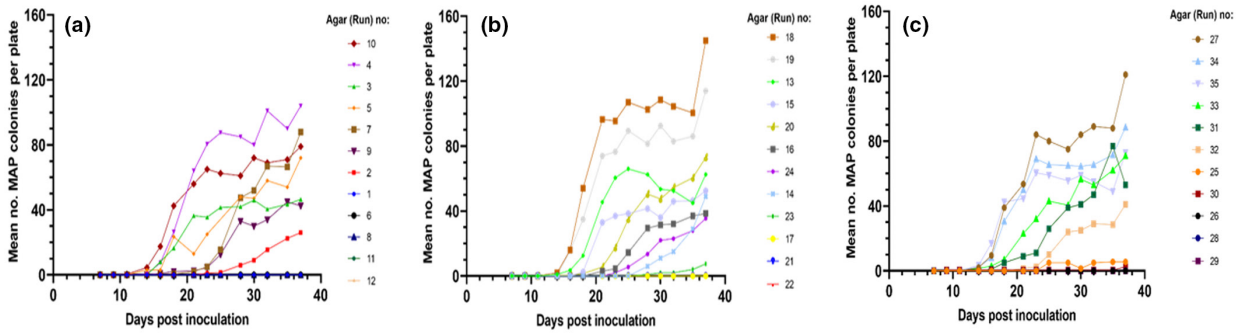

FIGURE 1 Growth curves for *Mycobacterium avium* subsp. *paratuberculosis* (MAP) 796 PSS on 35 different formulations of agar (runs) tested during the fractional factorial design experiment. (a) Runs 1–12, (b) 13–24 and (c) 25–35. Figure legends list agars from the best to the worst growth in each case. Details of the composition of each agar (run) are provided in Table S1

TABLE 1 Growth of MAP in triplicate plates (Reps 1–3) of Middlebrook 7H9 agar supplemented with Tween 80 and other supplements (Mycobactin J and OADC or ADC) after 63 days of incubation (− indicates no visible growth, + indicates <100 colonies, ++ indicates ≥100 colonies)

	7H9/Mycobactin J			7H9/OADC/Mycobactin J			7H9/ADC/Mycobactin J		
Tween 80 (%)	**Rep 1**	**Rep 2**	**Rep 3**	**Rep 1**	**Rep 2**	**Rep 3**	**Rep 1**	**Rep 2**	**Rep 3**
0	−	−	−	+	+	−	−	−	−
0.001	−	−	−	+	+	−	+	+	+
0.01	−	−	−	+	+	−	−	+	+
0.1	−	+	+	+	+	+	++	++	++
1	−	−	−	−	−	−	++	++	++

inputted into the Design Expert 13 software, which fitted the data to linear or quadratic models, and used these models to calculate an optimized medium composition based on the desired MAP growth responses. Variables from the fractional factorial experiment were analysed visually using the Pareto Chart of Standardized Effects and Half-Normal Plot to identify the factors with the greatest effect on the response variables. For the Half-normal Plot, the normality of the data was confirmed using the Shapiro–Wilk test. Factors that were identified as potentially significant from the Pareto Chart of Standardized Effects and Half-Normal Plot were confirmed using ANOVA. The models from the RSM experiment were analysed using ANOVA, with the models and the model variables being considered significant at $p < 0.05$.

The predictive performance of the models was validated by evaluating lack of fit, coefficient of regression and adequate precision. Lack of fit is considered significant at $p < 0.05$, with a nonsignificant lack of fit being a positive model metric that indicates the model is a good fit for the data. For coefficient regression, Predicted R^2 measures how well a regression model predicts responses for new observations, while adjusted R^2 measures model fit adjusted by the number of independent variables (as models with more variables will appear to be a better fit, even if they are unable to make accurate predictions). In general, the predicted R^2 and adjusted R^2 should be within 0.2 of each other (Stat-Ease®, 2021c). Finally, the Adequate Precision ratio measures the signal-to-noise ratio and should be >4.

The assumptions of ANOVA were analysed visually using a range of diagnostic plots, including the normal plot of residuals, which verified the normality of the data, and the residuals versus predicted plot, which assessed the homoscedasticity of the variables. The residuals versus run plot checked that samples were drawn independently, and the predicted versus actual plot detected values that were not easily predicted by the model (Stat-Ease®, 2021a; Vining, 2010). Additionally, the Box-Cox plot determined whether the data required transformation and, when appropriate, validated the chosen transformation. The Cook's Distance, DFFITS versus Run, or DFBETAS versus Run plots checked for outliers, and the Leverage versus Run plot checked for significant deviations (Stat-Ease®, 2021b).

RESULTS

Initial screening of potential supplements

After 76 days, >100 MAP colonies were consistently observed on all agar plates supplemented with 15–25 mg ml^{-1} taurocholic acid. However, all plates supplemented with less or no taurocholic acid had <100 visible MAP colonies, and agar supplemented with 5 mg ml^{-1} had no visible growth in any replicate. These results indicated that taurocholic acid may have stimulated MAP growth at concentrations of 15 mg ml^{-1} or above.

Table 1 shows the results observed on 7H9/Mycobactin J agar supplemented with Tween 80 and no ADC or OADC, ADC only or OADC only after incubation for 63 days. The results of this experiment indicated that 0.1% (v/v) Tween 80 may be the optimal concentration for MAP culture since this was the only concentration that allowed some growth of MAP on 7H9/Mycobactin J agar without additional supplements and promoted growth of MAP on all repeats containing 7H9/OADC/Mycobactin J agar. Interestingly, greater numbers of MAP colonies were observed on 7H9/ADC/Mycobactin J agar compared to 7H9/OADC/Mycobactin J agar when both were supplemented with 0.1% Tween 80. Also, colony numbers were high on 7H9/ADC/Mycobactin J agar containing 1% Tween 80, but there was no growth on 7H9/OADC/Mycobactin J agar with 1% Tween 80. For this reason, ADC was used to supplement the medium instead of OADC during the subsequent fractional factorial experiment.

Fractional factorial design experiment

The aim of this experiment was to statistically determine the main effects and interactions between the six factors

positively affecting the growth of MAP: Tween 80, taurocholic acid, Mycobactin J, agar, casitone and egg yolk. A half-fraction two-level factorial design with three central points was selected as being most appropriate for this purpose, as a full factorial design or an RSM design would have required too many resources (Trutna et al., 2013). Figure 1A–C show growth curves for each of the 35 agars (runs). In most cases, runs with colonies that appeared earlier also had a greater final colony count. MAP colonies were first observed after 13 days on agar 10 (average of two repeats), and the highest number of colonies was observed on agar 18, which had an average of 145 cfu/plate. Agars 10 and 18 both contained 1 μg ml^{-1} Mycobactin J, 1.5% agar and 0.1% casitone and did not contain taurocholic acid (see Table S1). However, while run 10 was supplemented with 0.2% Tween 80 and did not contain egg yolk, run 18 was supplemented with 1% Tween 80 and 10% egg yolk. There were 10 agars (runs) that showed no evidence of visible MAP colonies; agars (runs) 1, 6, 8, 11, 12, 17, 21, 26, 28 and 29. The composition of these agars varied, but nine out of 10 were supplemented with 1% Tween 80, and all but one were not supplemented with egg yolk.

Two different responses were measured to create statistical models: *R*1 – time (days) to first visible MAP colonies and *R*2 – colony count on day 37, which was considered the end of the half-fractional factorial design experiment. The Pareto charts for *R*1 and *R*2 are shown in Figure 2. The coefficient estimates are shown in Table 2 and the results of ANOVA are provided in the Table S3. Both repeats of agar (run) 22 became overgrown with contaminants before MAP colonies became visible, and as a result, this run was excluded from the statistical modelling. As shown in Figure 2A, six factors were identified as having a significant effect on *R*1, three individual factors (A – Tween 80, B – taurocholic acid and F – egg yolk, all $p < 0.0001$), two sets of two-factor interactions (AF, *p*-value 0.0002 and BF, *p*-value 0.01), and one three-factor interaction (ABF, *p*-value 0.0001). Of these six factors, five also had a significant effect on *R*2 as shown in Figure 2B (A, B and F $p < 0.0001$; AF, *p* value 0.0022 and ABF, *p* value 0.0004), while factor BF was almost significant (*p* value 0.055). Usually, three-factor interactions are ignored, but in this case, the ABF interaction was deemed significant as it

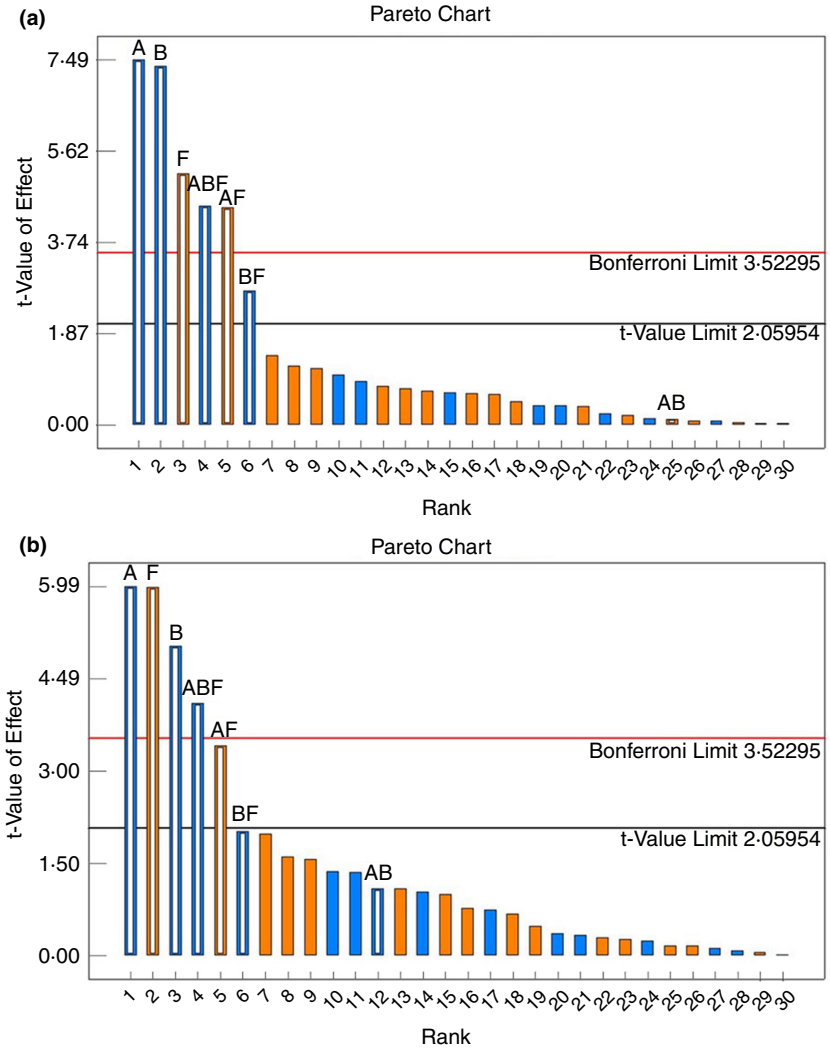

FIGURE 2 Pareto charts used for manual selection of significant factors after fractional factorial design experiment: (a) response 1 (number of days until first *Mycobacterium avium* subsp. *paratuberculosis* (MAP) colonies became visible) and (b) response 2 (average cfu/100 μl inoculum after 37 days of incubation). On both graphs, A = Tween 80, B = Taurocholic acid, and F = egg yolk. Orange bars indicate a positive effect of the factor, or combination of factors, on MAP growth (i.e. MAP colonies became visible earlier), while blue bars indicate a negative effect (i.e. MAP colonies took longer to become visible)

TABLE 2 Coefficient parameters for half-fraction two-level factorial design

	R1: Time to first visible colonies		R2: No. colonies after 37 days	
Parameters	**Coefficients**	**df**	**Coefficients**	**df**
Intercept	0.04551	1	5.751813	1
A-Tween 80	−0.01117	1	−1.77311	1
B-Taurocholic acid	−0.01096	1	−1.48419	1
F-Egg yolk	0.007687	1	1.767568	1
AB	0.000182	1	−0.32223	1
AF	0.006652	1	1.006973	1
BF	−0.00409	1	−0.59479	1
ABF	−0.00669	1	−1.21163	1
Centre Point	−0.00558	1	−1.91646	1

was above the Bonferroni limit for both responses. One additional hierarchical term (AB) was added after manual regression to support hierarchy and was confirmed to have an insignificant effect on both *R*1 (*p*-value 0.90) and *R*2 (*p*-value 0.29) with ANOVA. Of the factors that were identified as significant, only egg yolk (F) or Tween 80 in combination with egg yolk (AF) had a positive effect on MAP growth, and Tween 80 alone (A) or any interaction that included taurocholic acid (B, BF, ABF) had a negative effect. Hence, taurocholic acid was not studied further.

Fractional factorial design – Model fitting and validation

The Shapiro–Wilk results for half-normal plots were satisfactory with W values of >0.95 and p values of >0.75 for both MAP growth responses. The results of the ANOVA indicated that models for both responses (*R*1 – time to first visible colonies, *R*2 – no. colonies after 37 days) were significant ($p < 0.0001$). The lack of fit test was not significant for either response variable (*p*-values 0.89 and 0.90, respectively). For both MAP growth responses, the predicted R^2 values ($R1 = 0.78$, $R2 = 0.70$) were in reasonable agreement with the adjusted R^2 values ($R1 = 0.85$, $R2 = 0.80$), and the Adequate Precision ratios of 11.8 (*R*1) and 10.9 (*R*2) were acceptable. The results for all diagnostic plots were satisfactory, confirming that our models met the assumptions of ANOVA.

Fractional factorial design – Medium composition optimization

To identify the optimal medium composition, the models generated in the fractional factorial experiment were used to identify the composition which would give the minimum value for *R*1 (days until MAP colonies became visible) and the maximum value for *R*2 (colony count on day 37). The highest-ranked medium formulations were computed to contain 0.62%–1% v/v Tween 80, 1.46–2 μg ml^{-1} Mycobactin J, 1%–1.45% w/v agar, 0.07%–0.1% w/v casitone, 9.8%–10% v/v egg yolk and no taurocholic acid. Desirability scores ranged from 0.86 to 0.91.

RSM design experiment

Finally, the effects of factors that had been identified as most significant for MAP growth in the fractional factorial experiment were investigated further using an RSM approach. Based on the previous results, Tween 80 and egg yolk were selected as variables for RSM. For the third variable, casitone was selected, as, although it was not considered a significant factor in the fractional factorial experiment, it had the strongest positive effect on both *R*1 and *R*2 after egg yolk (F) and Tween 80 with egg yolk (AF). Furthermore, the software predicted that all 10 most optimal medium compositions would include casitone at a concentration of greater than 0.05%. A categorical factor was added to test the effects of ADC or OADC supplementation. This comparison was not incorporated into the fractional factorial experiment as there would have been too many runs, and categorical factors could not be added to factorial experiments with centre points. The concentrations of mycobactin J (1.65 μg ml^{-1}) and bacteriological agar (1.4% w/v) adopted were based on the average of the top five medium compositions predicted by the fractional factorial experiment and these were kept consistent across all agars (runs).

Figure 3A–F show growth curves for each agar run. Results are broken down by supplementation with OADC (Figure 3A–C) or ADC (Figure 3D–F), and then by egg yolk concentration (no egg yolk [A and D], 5% egg yolk [B and E] or 10% egg yolk [C and F]), with the intention of differentiating the effect of major and minor supplements (Tween 80 and casitone). It is clear to see that in the absence of egg yolk supplementation growth of MAP was very poor (Figure 3A,D), and only agar (run) 40, which contained 0.2% Tween 80 and 0.1% casitone in addition to OADC supplement, showed substantial growth of MAP. Although in the absence of egg yolk MAP grew better on media supplemented with OADC, Figure 3B, C, E and F shows that, in the presence of 5% or 10% egg yolk, MAP growth was superior on agar supplemented with ADC. The earliest appearance of colonies on both agar replicates was recorded after 9 days incubation on agar (run) 3, which contained 1% Tween 80, no casitone, 10% egg yolk and 10% ADC (see Table S2).

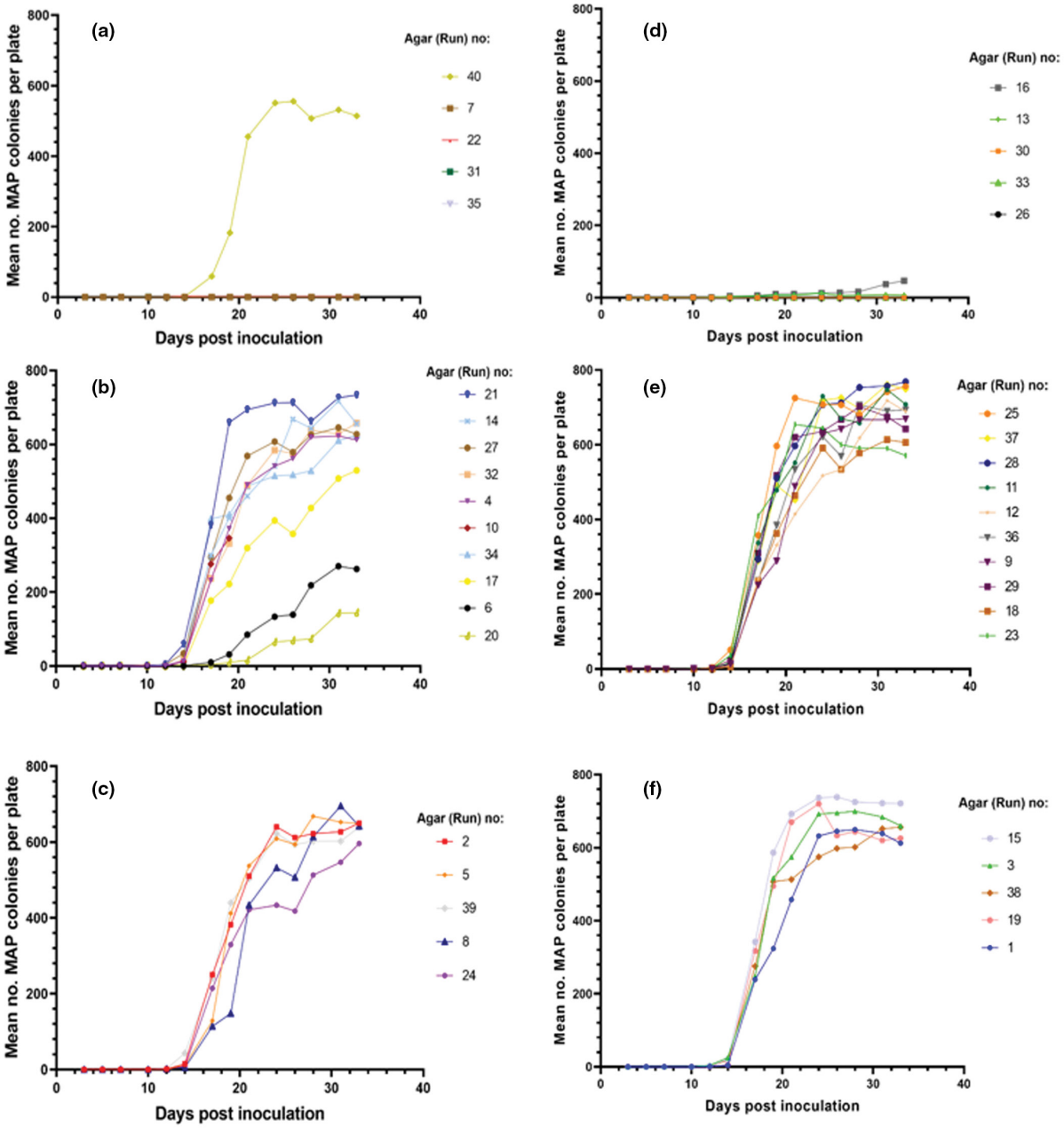

FIGURE 3 Growth curves for *Mycobacterium avium* subsp. *paratuberculosis* (MAP) 796 PSS on 40 different formulations of agar (runs) tested during the Response Surface Methodology experiment. Agars were supplemented with either 10% OADC (a–c) or 10% ADC (d–f) and no egg yolk (a, d), 5% egg yolk (b, e) or 10% egg yolk (c, f). Figure legends list agars from the best to the worst growth in each case. Details of the composition of each agar (run) are provided in Table S2

The highest number of colonies was recorded as 768 cfu/plate for agar (run) 28, which contained 0.6% Tween 80, 0.05% casitone, 5% egg yolk and 10% ADC. In contrast, the only factor that the six agars (runs 7, 26, 30, 31, 33 and 35) with no visible growth at the end of 40 days had in common was a lack of egg yolk further demonstrating the importance of this supplement.

For the RSM experiment, a third response, in addition to *R*1 and *R*2, was measured: *R*3 – time (days) taken for cultures to achieve maximal colony count, that is enter 'stationary phase'. This third response variable was introduced to gain a clearer understanding of the effect of each factor or combination of factors on the MAP growth rate, as values obtained for *R*1 could be skewed by clumps of MAP cells in the inoculum, or by how easy the colonies were to see on each type of medium. The significance of the effect of each factor on each of these responses was measured using ANOVA. The coefficient estimates are shown in Table 3 and the results are shown in Table S4. Run 10 was excluded from the analysis of *R*2 and *R*3 as

TABLE 3 Coefficient parameters for response surface methodology/central composite design

	R1: Time to first visible colonies		R2: No. colonies after 33 days		R3: Time to maximal colony count	
Factor	**Coefficients**	**df**	**Coefficients**	**df**	**Coefficients**	**df**
Intercept	0.0308	1	24.39	1	25.94	1
A-Tween 80	0.0023	1	−1.15	1	3.10	1
B-Casitone	0.0021	1	2.24	1	−3.75	1
C-Egg yolk	0.0085	1	10.83	1	−12.30	1
D-Supplement	−0.0010	1	−0.5215	1	0.9984	1
AB	−0.0021	1	−1.63	1	4.00	1
AC	−0.0063	1	2.10	1	−3.88	1
AD	0.0004	1	−0.8291	1	0.1000	1
BC	−0.0010	1	−1.49	1	1.63	1
BD	0.0024	1	1.52	1	−3.25	1
CD	−0.0009	1	−0.9224	1	4.30	1
A^2	0.0001	1	1.37	1	0.5242	1
B^2	−0.0027	1	−1.16	1	5.27	1
C^2	−0.0074	1	−9.97	1	7.02	1

both repeats became overgrown with contamination before the final colony counts were taken. However, run 10 was included in the analysis of *R*1, as MAP colonies had become visible before the contaminants overgrew the culture.

In contrast with the findings from the fractional factorial experiment, different factors were identified as significant for each response. Egg yolk (factor C) was the only factor that had a consistently significant positive effect on all three responses ($p < 0.0001$ for all responses). The interaction between Tween 80 and egg yolk (factor AC) had a significant positive effect on *R*1 and *R*2 (*p*-values 0.0006 and 0.028, respectively), casitone (factor B) had a significant positive effect on *R*2 only (*p*-value 0.010), and the interaction between egg yolk and ADC/OADC (factor CD) had a significant negative effect on *R*3 only (*p*-value 0.030).

RSM design – Model fitting and validation

The results of ANOVA indicated that the models for all responses were highly significant ($p < 0.0001$). Lack of fit was significant for *R*1 (*p*-value 0.023) but not for *R*2 (*p*-value 0.11) or *R*3 (*p*-value 0.22). For both *R*1 and *R*3, the predicted R^2 values ($R1 = 0.63$, $R3 = 0.65$) were not as close to the adjusted R^2 values ($R1 = 0.18$, $R3 = 0.42$) as would normally be expected, indicating that the model may not be a good fit for the data and may not be as capable of predicting the results with new data. However, the predicted R^2 of 0.88 and the adjusted R^2 value of 0.72 were in reasonable agreement for *R*2. The adequate precision ratios of 9.8 (*R*1), 16.01 (*R*2) and 9.56 (*R*3) were acceptable for all three responses.

As with the fractional factorial experiment, the assumptions of ANOVA were validated using a range of diagnostic plots. In the case of *R*1, satisfactory results were obtained for the normal plot of residuals, the residuals versus run plot and the Cook's distance plot. However, the values for Leverage versus Run indicated that there were significant deviations and Run 22 was identified as a potential outlier in the residuals versus the predicted plot. Results for *R*2 and *R*3 were better, although Run 40 was identified as a potential outlier on the normal plot of residuals for *R*2.

Overall, the model for *R*1 was not as good a fit for the data as models for *R*2 or *R*3 and may not have been as capable of predicting the outcome with new media combinations. However, for the end-users, all three models are designed to be used together for simultaneous prediction of *R*1, *R*2 and *R*3 and to support decision-making, and using the models in this manner would improve the accuracy and usefulness of the predictions. These results are also reflected in Figures 1 and 3 for the fractional factorial and RSM experiments, respectively.

RSM design – Medium composition optimization

To identify the optimal medium composition, the models produced with the results of the RSM experiment were used to identify the medium composition which would

give the minimum value for *R*1 (days until first colonies became visible), the maximum value for *R*2 (colony count on day 33) and the minimum value for *R*3 (days until colony counts reached maximum and cultures entered 'stationary phase'). The most desirable medium compositions were predicted to be those with 10% v/v egg yolk, 1% v/v Tween 80, 0.016–0.022% w/v casitone and ADC as the supplement. The predicted optimum medium was similar to the predictions from the fractional factorial experiment except that the casitone concentrations were much lower. The first 10 predicted solutions had identical desirability scores of 0.938. The high desirability scores and the similarity to results from the fractional factorial experiment indicated that the models used were robust.

Comparison of the optimized agar with solid media currently used for MAP culture

The growth of four MAP strains was compared on HEYM, Middlebrook 7H9/OADC/Mycobactin J agar and the optimized medium by means of streak plates. Colonies started to become visible on the 7H9/OADC/Mycobactin J agar and the optimized medium after 1 week of incubation at 37°C, and on all media after 3 weeks incubation. Figure 4 shows the cultures after 27 days of incubation. Growth of all MAP strains was faster and more luxuriant on 7H9/OADC/Mycobactin J agar and the optimized medium compared to growth on HEYM, but differences between the 7H9/OADC/Mycobactin J agar and the optimized medium were less obvious and varied between strains and repeats, for example there were larger colonies of MAP ATCC 19698 and 796 PSS (Figure 4A,C) on the new medium, while MAP B4 appeared to grow slightly better on Middlebrook 7H9/OADC/Mycobactin J agar (Figure 4B).

DISCUSSION

MAP is a suspected zoonotic pathogen that causes Johne's disease in ruminants and has been linked to a range of human autoimmune disorders. As such, limiting the spread of MAP is desirable, but control measures are hampered by a lack of sensitive and specific MAP detection methods, particularly inadequate MAP culture methods. The aim of this study was to optimize the composition of a solid culture medium to achieve maximal growth of MAP strains. The medium optimization was carried out via three sequential experiments. A screening experiment was carried out to test the effects of potential new supplements (Tween 80 and taurocholic acid) on MAP growth prior to the selection of factors for the main RSM experiment. Both Tween 80 and taurocholic acid potentially stimulated MAP growth, although cultures with both supplements were given an unusually long incubation period due to COVID-19 restrictions, and this may have affected the results. Next, the main effects of, and interactions between, different concentrations of six factors (supplements) were measured with a fractional factorial design, with the intention of selecting those factors with the most significant positive effect on MAP growth responses for further investigation within the subsequent RSM experiment. Finally, an RSM face-centred CCD was utilized to test the effects of three factors (Tween 80, casitone and egg yolk) that had the greatest positive effect on MAP growth during the fractional factorial experiment, and a comparison of OADC versus ADC was included as a categorical factor. The results of the final experiment were used by the Design Expert 13 software to predict an optimized solid medium composition, which was: 4.7 g Middlebrook 7H9 broth, 10 ml Tween 80, 0.19 g casitone, 100 ml egg yolk, 100 ml ADC, 1.65 mg Mycobactin J and 14 g bacteriological agar/L. The new medium was demonstrated to be superior to HEYM for growth of multiple MAP strains, as colonies appeared 2 weeks earlier on the new medium compared to HEYM, but MAP growth rates on Middlebrook 7H9/OADC/Mycobactin J agar were similar to the new medium.

The concentration of egg yolk was the single most important factor that influenced both the colony development rate and the number of MAP colonies on agar plates during this study and was the only factor to have a consistently positive effect on all measured growth responses (R1, R2 and R3). This result was not surprising given that the concentration of egg yolk has frequently been reported to have a significant effect on MAP growth (Pozzato et al., 2011; Whitlock et al., 1991). Egg yolk is a rich source of carbon, energy and iron for bacteria, and it contains lecithin that prevents cell clumping (Merkal & Curran, 1974; Whittington et al., 2013), although which egg yolk constituent specifically causes the significant increase in MAP development rate is unclear (Whittington, 2020). However, the hypothesis that the inclusion of egg yolk in solid culture media increased MAP growth rates and luxuriance of colony growth was not supported by the comparison of the new medium with 7H9/OADC/Mycobactin J agar, as little to no difference in colony formation rate was observed. Some possible reasons for this include that the colour of the new medium may have caused smaller colonies to be overlooked, that differences in growth rates may have been less obvious when culturing the faster-growing laboratory-adapted strains of MAP, or the new solid medium may need further optimization.

The optimal concentrations of Tween 80 recorded in the screening experiment agreed with those published by Van

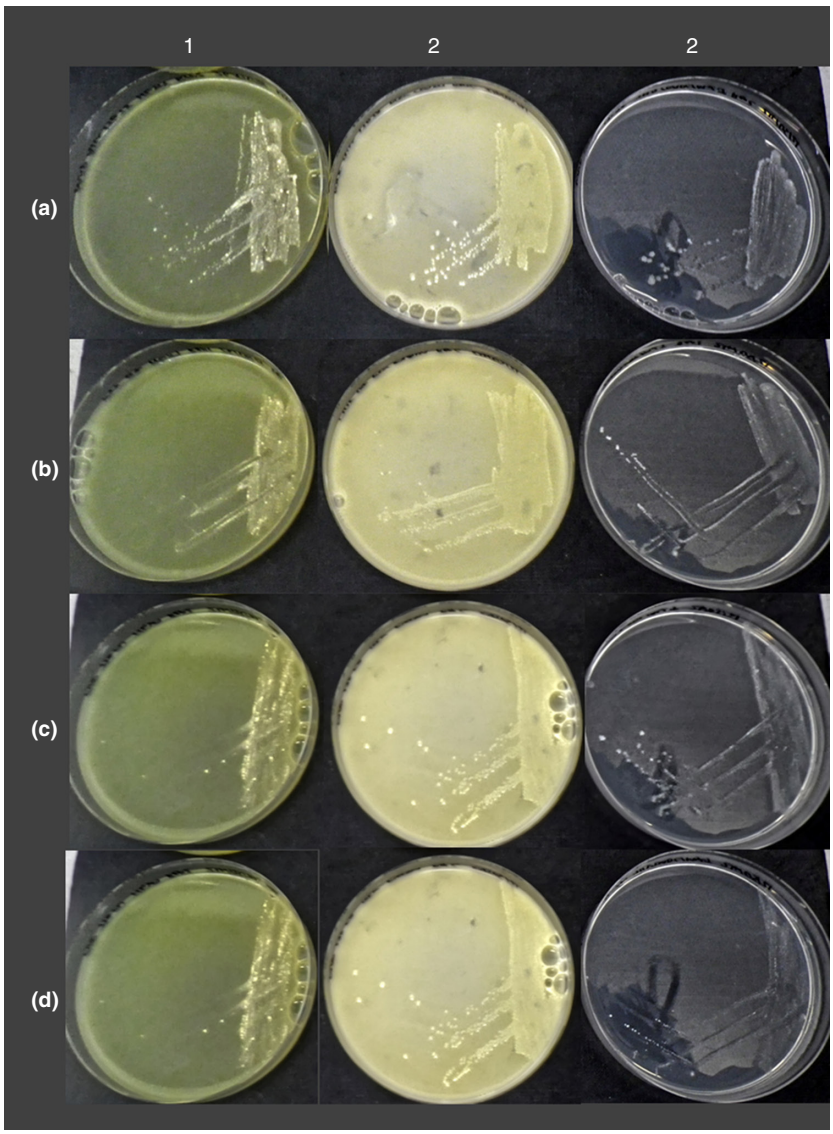

FIGURE 4 *Mycobacterium avium* subsp. *paratuberculosis* (MAP) cultures (10 μl) streaked on (1) Herrold's egg yolk mycobactin J agar (HEYM), (2) the optimized solid medium and (3) Middlebrook 7H9 agar with OADC and Mycobactin J, demonstrating more luxuriant growth on 2 after 27 days of incubation compared to 1. (a) MAP ATCC 19698, (b) MAP B4, (c) MAP 796 PSS and (d) MAP VIAS-Crohn's disease

Boxtel et al. (1990), who reported that 0.1% (v/v) Tween 80 was the optimal concentration for most of the strains of MAP tested, while a small number of strains grew optimally on agar containing 1% (v/v) Tween 80. Results from the fractional factorial and RSM experiments showed that increasing the concentration of Tween 80 from 0.2% v/v to 1% v/v had a negative effect on MAP growth responses, indicating that the lower concentrations of 0.1%–0.2% were optimal for MAP growth. This result supported previous reports that high concentrations of Tween 80 may inhibit the growth of mycobacteria (e.g. *Mycobacterium tuberculosis*, Sattler & Youmans, 1948). Therefore, it is quite surprising that the predicted optimal media from both the fractional factorial design and the RSM experiments contained 1% (v/v) Tween 80. However, the models used in this study predicted the best combination of factors rather than the best concentration of any single factor, and the higher concentration of Tween 80 (1%) may have been more beneficial than harmful for MAP growth when added in combination with 10% egg yolk and 10% ADC. In particular, the presence of egg yolk appeared to improve MAP tolerance for higher concentrations of Tween 80, as the agar run with the highest number of colonies from the fractional factorial experiment and the agar run with the earliest visible colonies from the RSM experiment were both supplemented with 1% Tween 80 and 10% egg yolk.

The results from the screening and RSM experiments indicated that ADC may support the growth of MAP cultures better than OADC, particularly in the presence of Tween 80. One possible explanation for this is that oleic acid is toxic to bacteria, particularly in high concentrations (Stinson & Solotorovsky, 1971), and ADC is identical to OADC apart from the absence of oleic acid, it may be less toxic for MAP. Tween 80 can be hydrolysed to oleic acid by some mycobacteria and has been reported to act as a nontoxic reservoir of this supplement (Stinson & Solotorovsky, 1971), thus ADC plus Tween 80 could potentially substitute for OADC. The results of the screening

experiment support this hypothesis, as MAP growth scores, were the highest on 7H9 agar containing ADC and 0.1%–1% Tween 80 compared to growth on agar containing ADC only, OADC and Tween 80 or OADC only. Results from the RSM experiment also supported this hypothesis, as although the combination of Tween 80 and ADC or OADC (factor AD) did not have a significant effect on the MAP growth responses, the final optimum medium predicted by the model contained 1% Tween 80 along with ADC, rather than OADC. Although OADC is used to supplement the majority of Middlebrook media used for MAP culture, ADC has been used in previous studies, for example Whittington et al. (1999) and Whittington et al. (2013) used ADC to supplement modified Middlebrook 7H10 agar and M7H9C broth, respectively. However, these studies did not state why ADC was used rather than OADC, or if there was any benefit in this substitution. The possible substitution of oleic acid (in OADC) with Tween 80 has not been highlighted before, thus is a novel finding of this study.

The effect of casitone on MAP growth responses was investigated because this supplement was included in two of the most recently developed MAP broth compositions (Pozzato et al., 2011; Whittington et al., 2013), although in both studies the specific effects of this supplement on MAP growth were not stated. In this study, casitone had a positive effect on all MAP growth responses, but the only significant effect was on the colony count after 33 days in the RSM experiment. This indicates that casitone may significantly increase the quantity of MAP growth, but not necessarily the growth rate. Despite the positive effect of casitone on MAP growth, the optimum medium predicted by the Design Expert 13 software contained very low concentrations of this supplement. This may be due to the high concentration of the other supplements in the agar. Casitone is a pancreatic digest of casein that provides bacteria with a source of peptides (Becton-Dickinson, 2017), so the presence of egg yolk in particular, as a potential protein source, may render this additional supplement unnecessary.

The predicted optimal concentration of Mycobactin J during the fractional factorial experiment was closer to the maximum value than the minimum, indicating that the higher concentration of 2 μg ml^{-1} was marginally superior to 1 μg ml^{-1}. This result was unsurprising, as 2 μg ml^{-1} has long been the recommended concentration of this supplement (Merkal, 1970). However, Pozzato et al. (2011) and Whittington et al. (2013) both supplemented broth with 1 μg ml^{-1} Mycobactin J, and as the concentration of Mycobactin J did not have a significant effect on MAP growth responses, reduction of the concentration to 1 μg ml^{-1} should not significantly affect MAP yields or growth rate. The concentrations of agar varied greatly within the top 10 predicted optimized media from the fractional factorial experiment. Our hypothesis was that lower concentrations of agar would improve MAP growth rates, but these results demonstrated that there was little benefit in changing the concentration of agar on MAP growth responses.

A major problem with MAP culture is the long incubation times required. Development of MAP colonies from isolates that had previously been cultured in vitro has been reported to take approximately 6–7 weeks before colonies become visible on HEYM (Pozzato et al., 2011; Whittington et al., 2011). Culture on the novel medium described here was more rapid, as 70% of the runs during the RSM experiment had visible MAP colonies after 2 weeks incubation. Time to the first detection of MAP on the new solid culture medium in this study was comparable to Pozzato's 7H9+ broth, which enabled the detection of 80% of positive samples after 2 weeks of incubation (Pozzato et al., 2011), or modified Middlebrook 7H10 or 7H11 agar, which enabled MAP colonies to become visible after 2–3 weeks (Whittington et al., 2011). However, the concentration of MAP in the inoculum used for the RSM experiment was higher than anticipated, which may have affected the observed colony development rate.

Limitations of this study include that only laboratory-adapted MAP strains were cultured on the new solid medium, and no antimicrobial supplements were included, so the suitability of the medium for primary isolation of MAP cannot be confirmed. Different strains of MAP were used for the screening experiments and the fractional factorial and RSM experiments. The type strain MAP ATCC 19698 was used in the screening experiments to determine the effect of each supplement on a typical MAP strain, while MAP 796 PSS, a strain originally isolated from pasteurized semi-skimmed milk (Grant et al., 2002) was used for the optimization of the new culture medium, as the medium was intended for the culture of MAP from milk samples specifically. Also, in the first experiment, agar was set either in slopes or in tissue culture flasks, while Petri dishes were used thereafter. This is unlikely to have had a significant effect on MAP growth given that MAP is regularly cultured on both slopes and Petri dishes, and the tissue culture flasks were very similar in size to the Petri dishes, but this does mean that the results may not be directly comparable. A final limitation was that the optimal concentration of each supplement was calculated between ranges that were preselected, so it is possible that concentrations outside those selected may be superior, for example the highest concentration of egg yolk tested in this experiment was 10%, but higher concentrations such as 16% (Pozzato et al., 2011) or 20% (Whittington et al., 1999; Whittington et al., 2013) may further increase MAP growth rates. To address these limitations, additional studies will be required.

The purpose of this study was to use factorial design and RSM for the optimization of a solid medium to be employed for MAP culture. This is the first study to report the use of RSM to optimize a culture medium specifically for the growth of MAP. Our results confirm that this is a valid alternative to the traditional 'one-supplement-at-a-time' approach to growth medium optimization. The advantage of being able to test multiple supplements in parallel is particularly beneficial given the long incubation times and expensive supplements (OADC, ADC and Mycobactin J) required for MAP culture. The final optimized solid culture medium used Middlebrook 7H9 broth as a base and contained 10 ml Tween 80, 0.19 g casitone, 100 ml egg yolk, 100 ml ADC, 1.65 mg Mycobactin J and 14 g bacteriological agar/L. The MAP strains used in this study have been subcultured and have adapted to grow in vitro, and therefore further studies will be necessary to confirm that the novel solid medium described here improves the growth of MAP upon primary isolation from veterinary samples.

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CONFLICT OF INTEREST

No conflict of interest was declared.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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