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Paper

# Evaluation of various formulations of Contagious Bovine pleuropneumonia vaccine (T1/44) for thermotolerance and shelf life

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## Abstract

Contagious Bovine Pleuropneumonia (CBPP) remains a significant livestock disease in sub-Saharan Africa, with the T1/44 vaccine's heat-lability and reliance on cold-chain infrastructure limiting its efficacy in tropical regions. This study evaluated the thermotolerance and shelf-life of seven CBPP vaccine formulations containing different stabilizing agents. Seven different vaccine formulations, incorporating stabilizing agents such as lactalbumin, casein, gelatin, skimmed milk, maltose, and sucrose, were tested. The formulations were subjected to accelerated stability testing at temperatures of 4°C, 25°C, and 37°C over a 14-day period. The viability of *Mycoplasma mycoides* was assessed by measuring colony-forming units (CFU) at each time point. Statistical analysis was performed using two-way ANOVA with a significance level of $p < 0.05$. All formulations showed a decrease in mycoplasma viability over time, with the most significant loss occurring at 37°C. Formulation 2 (Casein + Gelatin) showed the most promising results, with a stable mycoplasma count of $1.09\times10^7$ CFU/mL post-lyophilization and only minor reductions in CFU at various temperatures. After 14 days, Formulation 2 maintained the highest titres, with counts of $4.4\times10^7$ CFU/mL at 4°C, $1.2\times10^6$ CFU/mL at 25°C, and $8.0\times10^6$ CFU/mL at 37°C. Other formulations, such as Formulation 1 (Lactalbumin + Gelatin), Formulation 3 (Skimmed Milk), and Formulation 4 (Skimmed Milk + Maltose), showed moderate stability but experienced greater decreases in viability, particularly at elevated temperatures. For example, Formulation 3 (Skimmed Milk) had a significant decline in titre at 37°C, with counts falling to $1.05\times10^5$ CFU/mL by Day 14. Formulations 5, 6, and 7 (which included combinations of skimmed milk, sugars, and gelatin) showed poor stability at 37°C, with some formulations losing all viability at higher temperatures. This study highlights the effectiveness of casein and gelatin as stabilizers for enhancing the thermotolerance and shelf life of the T1/44 CBPP vaccine. These findings provide a valuable foundation for the development of more resilient CBPP vaccines that can be more widely used in resource-limited settings, especially in regions with unreliable cold-chain systems.

## Keywords

Stabiliser, CBPP vaccine, Thermo-stability, Shelf life, Mycoplasma mycoides

## Introduction

Contagious bovine pleuropneumonia (CBPP) is a notifiable respiratory disease of cattle, recognized by the World Organization for Animal Health. Endemic to sub-Saharan Africa, it leads to significant productivity losses due to high mortality and morbidity 1. Caused by *Mycoplasma mycoides* subsp. *mycoides* (Mmm), CBPP is characterized by severe fibrinous bronchopneumonia and pleural effusion during the acute to subacute stages, and by pulmonary sequestra in chronic cases. Additional lesions may also be found in the kidneys and in the carpal and tarsal joints of calves 2. It is estimated that CBPP causes annual losses of 30.1 million euros in twelve endemically infected sub-



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Saharan African countries 3. The financial implications of CBPP are particularly evident when eradication strategies are implemented, as shown in the 1995 outbreak in Ngami land, Botswana, where the cost of eradicating the disease was approximately US$97.5 million . The disease poses a serious threat to food security in endemic areas and is classified as a major respiratory ailment by the OIE/WOAH 4 5. It remains the only bacterial disease listed under the previous "List A" of diseases requiring immediate outbreak notification 6. Following the global eradication of rinderpest, the OIE ranks CBPP as the second most important transboundary animal disease affecting cattle 3.

Vaccination programs are one of the tools to achieve the CBPP free country or zone, along with proper animal identification, improved movement control, regular animal disease reports, active surveillance, authorized and exceptional treatment of clinical cases, and slaughter, in a coordinated strategy 7. Currently, the T1/44 and T1sr strains are used for CBPP prevention; however, these vaccines are thermally unstable and require storage at -20°C from manufacture until application 8. The efficacy of these vaccines is often compromised due to issues related to thermotolerance and shelf life, especially in many African countries, where maintaining a reliable cold chain is challenging. Fluctuations in temperature during transportation and storage can cause the vaccine to lose potency 9. Therefore, improving the stability of CBPP vaccines is crucial to ensure their effectiveness in the field, particularly in regions with inadequate infrastructure.

Developing thermotolerant vaccines that can be tested for long-term effectiveness would not only lower the cost of vaccine access for rural communities globally but also enhance immunity against diseases 10. Such improvements would alleviate some of the negative impacts on livestock production in sub-Saharan Africa. These advancements could potentially lead to more stable CBPP vaccines, which are ideally preserved with a matching thermo-stabilizer. Current vaccine requirements mandate that vaccines with attenuated T1/44 and T1sr strains contain at least $10^8$ mycoplasmas per dose, although the minimum acceptable is $10^7$ (WOAH, 2011). Efforts to improve the mycoplasma content per dose are ongoing, as some African manufacturers have struggled to meet the $10^8$ threshold 11. This initiative also aims to address production, storage, and transportation challenges that have hindered global vaccine viability goals.

This study aims to evaluate the potential of various formulations of the CBPP (T1/44) vaccine with different stabilizers to enhance its thermotolerance and shelf life, ensuring its effectiveness under diverse storage and distribution conditions commonly found in sub-Saharan Africa, particularly in areas where refrigeration is limited. This study is guided by two main inquiries: (1) Which stabilizers can maintain the viability and potency of the CBPP (T1/44) vaccine at high temperatures, ensuring its effectiveness in regions with limited refrigeration? (2) How do different stabilizers affect the potency and shelf life of the vaccine over time? The findings would provide valuable insights to improve vaccine formulations, making them more accessible and effective, particularly in resource-limited areas.

# *Materials and methods*

## Preparation of CBPP T1/44 strain medium

One (1) litre of HEPES buffered medium was prepared according to the African Union Pan Africa Veterinary Vaccine Centre (AU-PANVAC) protocol by weighing and mixing essential ingredients namely; Tryptose (20 g), Yeast extract (5 mL), Glucose (5 g), Glycerol (5 mL), Sodium Chloride (NaCl, 5 g), Di-sodium Hydrogen Orthophosphate ($Na_2HOP_4$ 2.5 g) into 750 mL of distilled water. The mixture was then sterilized at 121°C for 15 minutes. After sterilization, penicillin (4 g) was filtered using 0.22-micron filters and added to the medium to preserve its sterility and potency. The medium was then kept in an incubator to observe any changes in colour and turbidity. In order to allow the HEPES medium to have growth ability of MmmSC, 50 mL HEPES and 200 mL Horse serum (20%) were added. Given that Mycoplasma develops best at a pH of 7.0 to 8.0, with pH 7.4 being the ideal range, and that a pH of less than 6.5 results in a halted growth and rapid cell death (Waite and March, 2002), the pH was adjusted by adding a few drops (4.6 mL) of 1N NaOH. The complete medium was then left to stay in an incubator at 37°C for 24 hours to check for sterility through colour change.

## Preparation of Stabilizers

The CBPP vaccine T1/44 was formulated with seven different stabilizers, which were prepared using the protocol from AU-PANVAC Standard Operating Procedures (SOPs). These stabilizers, consisting of proteins, polysaccharides, or a combination of both (Table 1), were separately weighed and mixed. After preparation, the stabilizers were sterilized at 121°C for 15 minutes and then stored in a 37°C incubator.



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| Formulations | Components | Percentages |
|---|---|---|
| (1) | Skimmed Milk + Maltose | 80% + 13% |
| (2) | Casein + Gelatin | 80% + 13% |
| (3) | Skimmed Milk | 16% |
| (4) | Skimmed Milk + Maltose | 7.5% + 7% |
| (5) | Skimmed Milk + Maltose + Gelatin | 7.5% + 7% + 13% |
| (6) | Skimmed Milk + Sucrose | 7.5% + 7% |
| (7) | Skimmed Milk + Sucrose + Gelatin | 7.5% + 7% + 13% |

**Table** I. Formulation of the seven different stabilizers used in the production of the CBPP T1/44 vaccine.

## Mycoplasma culture

A 100 mL aliquot of HEPES buffered medium was inoculated with a reconstituted freeze-dried vial of the Mmm vaccine seed strain T1/44, supplied by the African Union Pan African Veterinary Vaccine Centre. The vaccine strain was reconstituted with 2 mL of normal saline at a seed ratio of 2%. The inoculated medium was then incubated at 37°C for 72 hours to allow for appropriate mycoplasma growth, as determined by visual inspection of the culture turbidity (swirling cloud formation).

After incubation, a few drops of the inoculum were transferred into Tryptic Soy Broth (TSB), Fluid Thioglycollate Medium (FTM), and Blood Agar to check for microbial growth. TSB was incubated at room temperature (20-25°C), while FTM and Blood Agar were incubated in a $CO_2$ incubator at 37°C for 14 days. During the observation period, clear colour was maintained and no signs of turbidity was recorded.

## Vaccine Constituent Blending

This stage forms the final bulks. Each formulation (1-7) contained 120 millilitres of the stabilizers and 120 mL of the Mycoplasma culture were mixed in equal proportions (1:1). Two mL of each vaccine formulations were aseptically saved, before lyophilization, for immediate titration. The remaining 238 mL per vaccine were aliquoted (2 mL per aliquot) into glass vials for subsequent lyophilization.

## Filling and half stoppering

The CBPP T1/44 vaccine culture was automatically filled into sterile, dehydrogenated glass vials by a machine after being combined with the required stabilizers. The lyophilization process was carried out using a Labconco FreeZone 6 Liter Benchtop Freeze Dry System (USA), with the following operating parameters: condenser temperature of −50°C, pressure of 0.04 mbar, and a 48-hour cycle. Each glass vial contained two millilitres (2mL) of each of the seven experimentally blended vaccine formulations. Following the lyophilization process, the vials were labelled, sealed, and stored at −20°C. The residual moisture test for each vaccine formulation showed varying results: Formulation 1 had 2.18%, Formulation 2 had 2.78%, Formulation 3 had 1.52%, Formulation 4 had 3.20%, Formulation 5 had 3.23%, Formulation 6 had 2.23%, and Formulation 7 had 3.63% residual moisture.

## Mycoplasma titration

Bacterial viability was measured through titration to assess stability after reconstituting each exposed vial. Accelerated stability studies were conducted on lyophilized formulations (four vials, each containing 2 mL, at each temperature and time point) to evaluate their viability after storage at 4°C, 25°C, and 37°C for 1, 3, 7, 10, and 14 days. Stability was quantified by assessing the reduction of viable bacteria within vaccine vials for each formulation. The exposed vials were reconstituted with 2 mL of normal saline, followed by culture on complete HEPES-buffered medium to enumerate the remaining viable mycoplasmas. Each formulation was tested in two independent experiments (n=2). For each



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experiment, both pre-and post- lyophilization were determined, and titrations were performed in duplicate. Titration of all seven (7) formulations was performed in six ten-fold serial dilutions, and 25 microliters were dropped onto the tryptose agar plate, followed by incubation at 37°C, 5% $CO_2$ for seven days. Inoculated Tryptose Agar (TA) plates were examined under an inverted microscope at x40 magnification using a Stemi 305 Zeiss microscope. The colonies, characterized by their 'fried egg' appearance, were counted, and the results were recorded. The final vaccine titre was determined using the Spearman-Karber formula, based on the geometric mean calculated between titrations performed on solid and liquid media.

## Data Analysis

All experiments were conducted according to the standard operation procedures, and all raw data were recorded on structured laboratory log sheets. The obtained results were electronically filed in Microsoft Excel 2019 and statistical analysis was done using Microsoft Excel and STATA (Version 14.2, StataCorp LLC, Texas, USA). The level of significance for this experiment was $p < 0.05$, at 5% significance. The output of the data analysis was presented in tables, graphs, and statistical summaries.

# *Results*

## Mycoplasma counts in the wet and post- lyophilized CBPP T1/44 vaccines

The average number of viable organisms in the wet vaccines for each stabilizer formulation was as follows: Formulation 1 (Lactalbumin + Gelatin) had $10^{8.68}$ CFU/mL, Formulation 2 (Casein + Gelatin) had $10^{8.08}$ CFU/mL, Formulation 3 (Skimmed Milk) had $10^{9.56}$ CFU/mL, Formulation 4 (Skimmed Milk + Maltose) had $10^{9.27}$ CFU/mL, Formulation 5 (Skimmed Milk + Maltose + Gelatin) had $10^{8.20}$ CFU/mL, Formulation 6 (Skimmed Milk + Sucrose) had $10^{8.94}$ CFU/mL, and Formulation 7 (Skimmed Milk + Sucrose + Gelatin) had $10^{8.40}$ CFU/mL (Figure 1). After lyophilization, the average titres for the formulations were: $10^{8.51}$ CFU/mL for Formulation 1, $10^{9.57}$ CFU/mL for Formulation 2, $10^{9.36}$ CFU/mL for Formulation 3, $10^{9.07}$ CFU/mL for Formulation 4, $10^{9.12}$CFU/mL for Formulation 5, $10^{8.81}$ CFU/mL for Formulation 6, and $10^{8.90}$ CFU/mL for Formulation 7.

Following the approach outlined by Sangdjinan et al., (2022) titre losses are expressed as differences in exponents. Lyophilization resulted in the following titre losses: Formulation 1 (Lactalbumin + Gelatin) showed a loss of $10^{0.17}$ CFU/mL, Formulation 2 (Casein + Gelatin) lost $10^{-1.49}$ CFU/mL, Formulation 3 (Skimmed Milk) lost $10^{0.2}$ CFU/mL, Formulation 4 (Skimmed Milk + Maltose) lost $10^{0.2}$ CFU/mL, Formulation 5 (Skimmed Milk + Maltose + Gelatin) lost $10^{-0.92}$ CFU/mL, Formulation 6 (Skimmed Milk + Sucrose) lost $10^{0.13}$ CFU/mL, and Formulation 7 (Skimmed Milk + Sucrose + Gelatin) lost $10^{-0.5}$ CFU/mL. Formulations 2, 5, and 7, which contained gelatin, showed an increase in titre values of $10^{1.49}$ CFU/mL, $10^{0.92}$ CFU/mL, and $10^{0.5}$ CFU/mL for Formulations 2, 5, and 7, respectively, while the other formulations experienced a gradual decrease in titre. Although Formulation 2 showed the highest increase in titre ($10^{1.49}$ CFU/mL) (Figure 1) there was no statistically significant difference in titre loss among the formulations. Nevertheless, Formulation 2's stabilizer was identified as the most effective in preserving vaccine viability.

## CBPP Vaccines Titre Stability Over Time

The result obtained after testing the seven vaccine formulations by 6, 10-fold serial dilutions before and after lyophilization under storage conditions of 4°C, 25°C, and 37°C showed a progressive decline in titres over time, but with degradation rates that varied markedly depending on the formulation and storage temperature.



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**Figure** 1. Pre- and post-lyophilization titres of CBPP T1/44 vaccines across stabilizers.

## Viability and stability of the vaccine formulations on day 3

On Day 3, the viability and stability of each of the seven vaccine formulations with various stabilizers showed variability under different temperature conditions (Figure 2). In Formulation 1, the bacterial titre decreased from $10^{8.51}$ CFU/mL (post-lyophilization) to $10^{8.08}$ CFU/mL at 4°C, $10^{7.60}$ CFU/mL at 25°C, and $10^{6.60}$ CFU/mL at 37°C. Formulation 2 showed a decrease from $10^{9.57}$ CFU/mL (post-lyophilization) to $10^{8.88}$ CFU/mL at 4°C, $10^{7.90}$ CFU/mL at 25°C, and $10^{8.45}$ CFU/mL at 37°C. In Formulation 3, the titre dropped from $10^{9.36}$ CFU/mL (post-lyophilization) to $10^{8.51}$ CFU/mL at 4°C, $10^{8.20}$ CFU/mL at 25°C, and $10^{6.6}$ CFU/mL at 37°C. Formulation 4 decreased from $10^{9.07}$ CFU/mL (post-lyophilization) to $10^{8.20}$ CFU/mL at 4°C, $10^{7.60}$ CFU/mL at 25°C, and $10^{7.00}$ CFU/mL at 37°C. Formulation 5, with an initial titre of $10^{9.12}$ CFU/mL, decreased to $10^{7.90}$ CFU/mL at 4°C, $10^{7.60}$ CFU/mL at 25°C, and $10^{5.60}$ CFU/mL at 37°C. Formulation 6 dropped from $10^{8.81}$ CFU/mL (post-lyophilization) to $10^{7.90}$ CFU/mL at 4°C, $10^{7.60}$ CFU/mL at 25°C, and $10^{4.90}$ CFU/mL at 37°C. Finally, Formulation 7 decreased from $10^{8.90}$ CFU/mL (post-lyophilization) to $10^{7.90}$ CFU/mL at 4°C, $10^{7.60}$ CFU/mL at 25°C, and reached 0 CFU/mL at 37°C. These results demonstrate that all formulations remained stable at 4°C with only minor losses in titre. However, at 25°C and 37°C, more significant losses were observed, with Formulation 7 showing a complete loss of viability at 37°C.

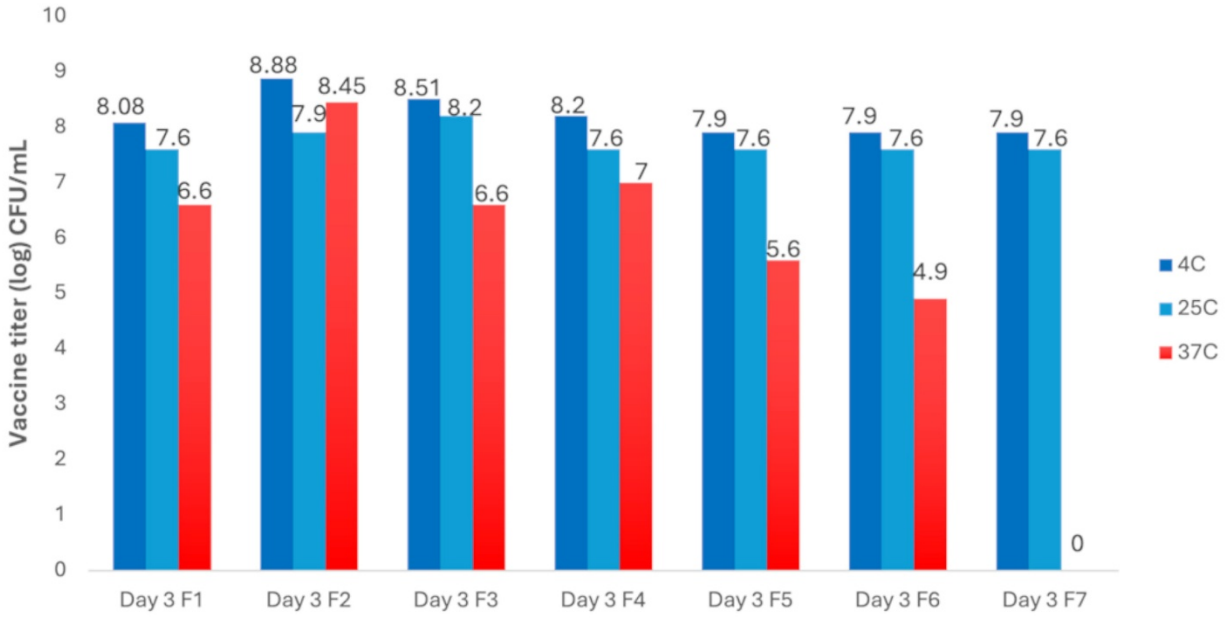


**Figure** 2. Residual titres (CFU/mL) of CBPP T1/44 formulations 1 to 7 on Day 3.



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## Viability and stability of the vaccine formulations on Day 7

On Day 7, the bacterial titres in all seven vaccine formulations showed a decline in viability, with significant losses observed at higher temperatures (Figures 3). In Formulation 1, the titre decreased from $10^{8.51}$ CFU/mL (post-lyophilization) to $10^{7.90}$ CFU/mL at 4°C, $10^{7.08}$ CFU/mL at 25°C, and $10^{4.60}$ CFU/mL at 37°C. Formulation 2 decreased from $10^{9.57}$ CFU/mL (post-lyophilization) to $10^{8.45}$ CFU/mL at 40°C, $10^{6.81}$ CFU/mL at 25°C, and $10^{7.90}$ CFU/mL at 37°C. Formulation 3 decreased from $10^{9.36}$ CFU/mL (post-lyophilization) to $10^{8.08}$ CFU/mL at 4°C, $10^{7.60}$ CFU/mL at 25°C, and $10^{7.37}$ CFU/mL at 37°C. In Formulation 4, the titre dropped from $10^{9.07}$ CFU/mL (post-lyophilization) to $10^{7.23}$ CFU/mL at 4°C and $10^{6.86}$ CFU/mL at 25°C. Formulation 5 decreased from $10^{9.12}$ CFU/mL (post-lyophilization) to $10^{7.60}$ CFU/mL at 4°C and $10^{6.80}$ CFU/mL at 25°C. Formulation 6 dropped from $10^{8.81}$ CFU/mL (post-lyophilization) to $10^{7.30}$ CFU/mL at 4°C and $10^{6.20}$ CFU/mL at 25°C. Finally, Formulation 7 decreased from $10^{8.90}$ CFU/mL (post-lyophilization) to $10^{7.83}$ CFU/mL at 4°C and $10^{6.50}$ CFU/mL at 25°C. However, at 37°C, formulations 4, 5, 6, and 7 reached zero (0) CFU/mL by Day 7, indicating a complete loss of viability under these conditions.

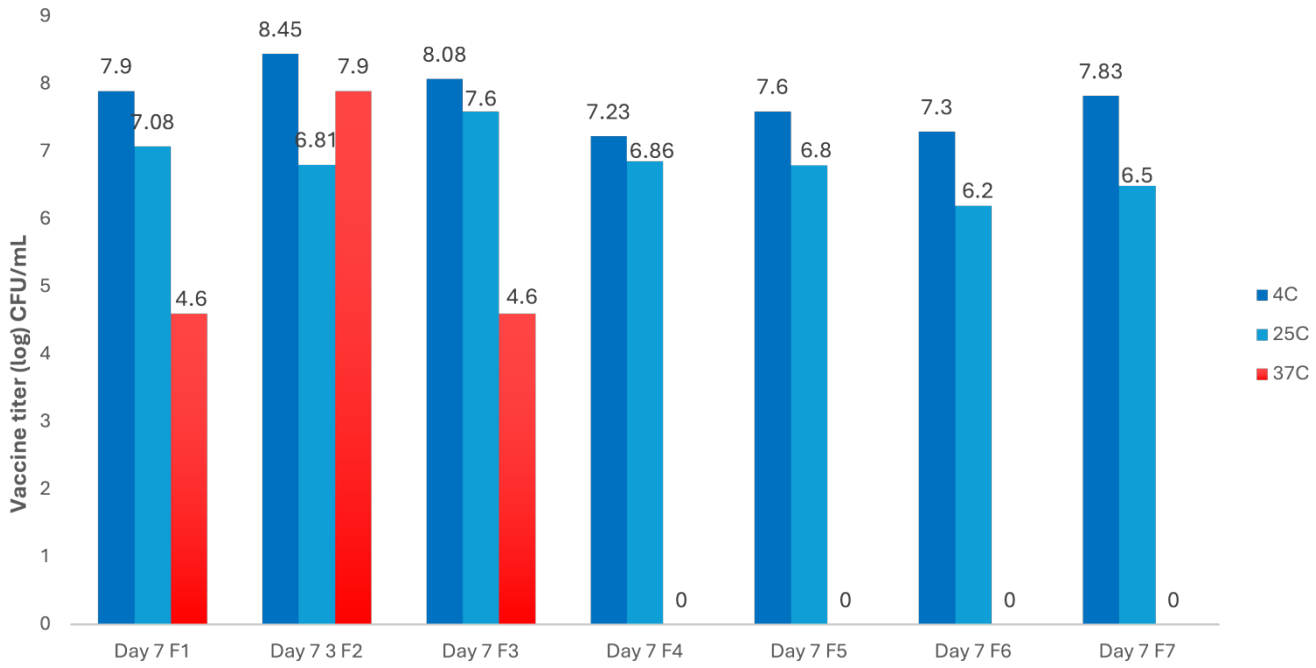


**Figure** 3. Residual titres (CFU/mL) of CBPP T1/44 formulations 1 to 7 at Day 7.

## Viability and stability of the vaccine formulations on Day 10

On Day 10 of storage, all vaccine formulations except Formulation 2 showed a complete loss of viability at 37°C, with titres dropping to zero (0) CFU/mL (Figure 4). Formulation 2, however, maintained higher titres at 4°C while all formulations experienced a significant decline at 25°C. Specifically, the bacterial titre in Formulation 1 decreased from $10^{8.51}$ CFU/mL (post-lyophilization) to $10^{7.60}$ CFU/mL at 4°C, and $10^{6.08}$ CFU/mL at 25°C. Formulation 2 decreased from $10^{9.57}$ CFU/mL (post-lyophilization) to $10^{8.00}$ CFU/mL at 4°C, $10^{6.30}$ CFU/mL at 25°C, and $10^{7.38}$ CFU/mL at 37°C. In Formulation 3, the titre dropped from $10^{9.36}$ CFU/mL (post-lyophilization) to $10^{7.60}$ CFU/mL at 4°C and $10^{6.30}$ CFU/mL at 25°C. Formulation 4 decreased from $10^{9.07}$ CFU/mL (post-lyophilization) to $10^{7.00}$ CFU/mL at 4°C and $10^{5.51}$ CFU/mL at 25°C. Formulation 5 dropped from $10^{9.12}$ CFU/mL (post-lyophilization) to $10^{6.60}$ CFU/mL at 4°C and $10^{5.60}$ CFU/mL at 25°C. In Formulation 6, the titre decreased from $10^{8.81}$ CFU/mL (post-lyophilization) to $10^{6.78}$ CFU/mL at 4°C and $10^{5.08}$ CFU/mL at 25°C. Finally, Formulation 7 decreased from $10^{8.90}$ CFU/mL (post-lyophilization) to $10^{6.51}$ CFU/mL at 4°C and $10^{5.30}$ CFU/mL at 25°C. These results demonstrate that while all formulations maintained higher titres at 4°C, they all showed significant losses at 25°C and 37°C, with only Formulation 2 exhibiting notable stability at 4°C.



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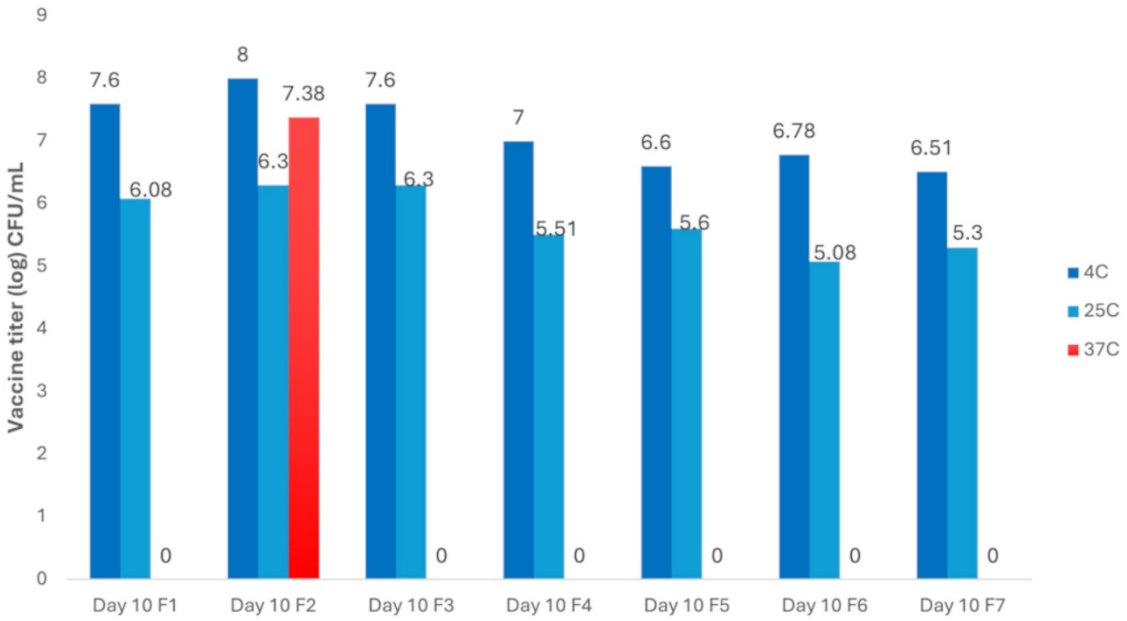


**Figure** 4. Residual titres (CFU/mL) of CBPP T1/44 formulations 1 to 7 at Day 10.

## Viability and stability of the vaccine formulations on Day 14

On Day 14 of storage, all vaccine formulations except Formulation 2 reached zero (0) CFU/mL at 37°C (Figure 5). Formulation 2 showed significant maintenance of titres at 4°C, while all the formulations experienced a sharp decline at 25°C. Specifically, the bacterial titre in Formulation 1 decreased from $10^{8.51}$ CFU/mL (post-lyophilization) to $10^{6.51}$ CFU/mL at 4°C and $10^{5.9}$ CFU/mL at 25°C. Formulation 2 decreased from $10^{9.57}$ CFU/mL (post-lyophilization) to $10^{7.64}$ CFU/mL at 4°C, $10^{6.08}$ CFU/mL at 25°C, and $10^{6.9}$ CFU/mL at 37°C. Formulation 3 decreased from $10^{9.36}$ CFU/mL (post-lyophilization) to $10^{6.2}$ CFU/mL at 4°C and $10^{5.9}$ CFU/mL at 25°C. Formulation 4 decreased from $10^{9.07}$ CFU/mL (post-lyophilization) to $10^{6.45}$ CFU/mL at 4°C and $10^{4.9}$ CFU/mL at 25°C. Formulation 5 decreased from $10^{9.12}$ CFU/mL (post-lyophilization) to $10^{6.4}$ CFU/mL at 4°C and $10^{4.9}$ CFU/mL at 25°C. Formulation 6 decreased from $10^{8.81}$ CFU/mL (post-lyophilization) to $10^{6.6}$ CFU/mL at 4°C and $10^{4.6}$ CFU/mL at 25°C. Finally, Formulation 7 decreased from $10^{8.90}$ CFU/mL (post-lyophilization) to $10^{6.3}$ CFU/mL at 4°C and $10^{5.08}$ CFU/mL at 25°C.

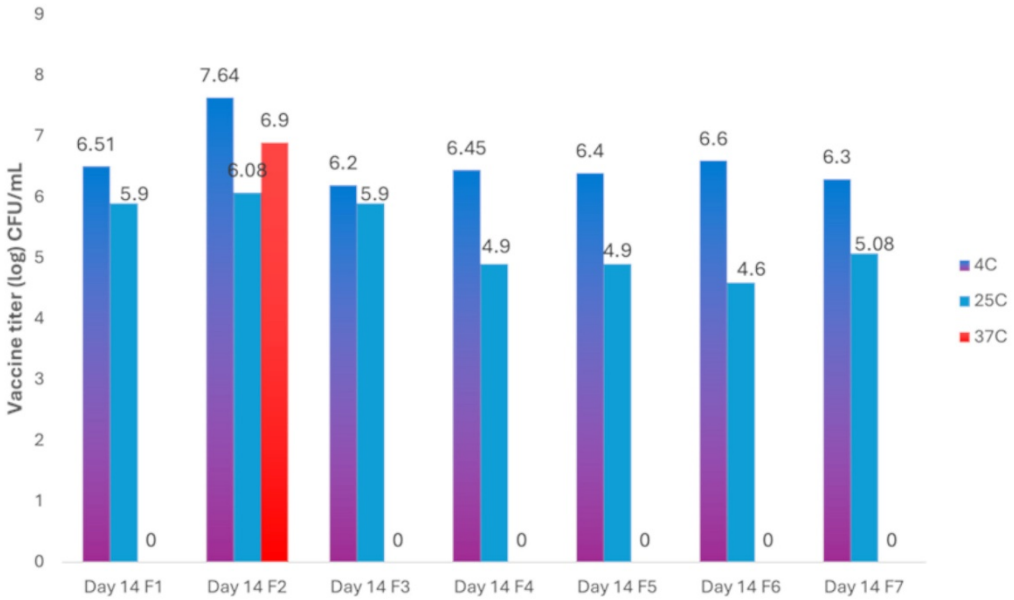


**Figure** 5. Residual titres (CFU/mL) of CBPP T1/44 formulations 1 to 7 at Day 14



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## Effects of Formulation, Temperature and Freezing on CBPP T1/44 Vaccine Titre

The Two-Way ANOVA analysis for the CBPP T1/44 vaccine titre examined the effects of formulation (sample) and temperature on the titre levels (Table 2). The results indicated that formulation did not have a significant effect on the titres, as the P-value (0.9028) was greater than 0.05. On the other hand, temperature had a highly significant effect on titres, with a very low P-value (3.95E-25), suggesting that temperature strongly impacts titre values. The interaction between formulation and temperature was not significant, as evidenced by a high P-value (0.9756), indicating that the effect of formulation on titres is independent of temperature. The within-group error variance (SS = 3149.53) represents the unexplained variation in the data.

In the One-Way ANOVA for pre and post freezing, the results showed that freezing did not significantly affect the titres, as the P-value (0.5873) was greater than 0.05. This indicates that there is no statistically significant difference in titres between the pre and post freezing groups.

| Source of Variation | Sum of Squares (SS) | Mean Square (MS) | F-value | P-value | F crit |
|---|---|---|---|---|---|
| **Two-Way ANOVA** | | | | | |
| Sample (Temperature x Day levels) | 23.23717 | 3.872861 | 0.361521 | 0.902839 | 2.129473 |
| Columns (Temperature & Day) | 1466.322 | 733.1609 | 68.43859 | 3.95E-25 | 3.026466 |
| Interaction (Temp x Day) | 46.47434 | 3.872861 | 0.361521 | 0.975577 | 1.785196 |
| Within (Error) | 3149.528 | 10.71268 | | | |
| Total | 4685.562 | | | | |
| **One-Way ANOVA (Pre and Post Freezing)** | | | | | |
| Between Groups | 1.210543 | 0.201757 | 0.820556 | 0.587284 | 3.865969 |
| Within Groups | 1.72115 | 0.245879 | | | |
| Total | 2.931693 | | | | |

**Table** II. ANOVA Analysis of the Effects of Formulation, Temperature, and Freezing on CBPP T1-44 Vaccine titre.

# *Discussion*

This study was conducted with the aim of evaluating the thermotolerance and shelf life of seven formulations of the CBPP vaccine T1/44 containing different stabilizing agents with a view to ascertaining their viability and potency. The thermotolerance evaluation showed persistent reduction in the titre values of the vaccine's antigen with most of the formulations as the temperature increased from 4°C to 25°C and 37°C indicating thermal instability. The thermal instability observed is an indication of poor effectiveness of the vaccines. Many live attenuated vaccines experience a significant loss of potency between manufacture and administration (Wang et al., 2020). This issue is especially problematic in sub-Saharan African countries, where limited and unreliable cold-chain infrastructure heightens the risk of vaccine failure, ultimately contributing to an increased disease burden (Schlehuber et al., 2011).

Vaccines are among the most cost-effective and impactful public health tools, preventing around three million deaths annually. However, hundreds of thousands of preventable deaths occur each year due to thermal instability in vaccine formulations (Dumpa et al., 2019). Vaccine stability, influenced by factors such as stabilizers, production processes, lyophilization techniques, cold-chain preservation and storage containers, is critical to addressing this challenge (Sangdjinan et al., 2022). Both human and animal vaccines, including CBPP vaccine, have been exposed to extreme temperatures in regions with poor cold-chain systems (Ashok et al., 2017; Nuvey et al., 2022). This calls for a formulation that is stable during storage and transportation. Thermotolerant vaccines with extended shelf life are essential to maximize the success rate of CBPP vaccination campaigns, especially under resource-limited settings.

The Two-Way ANOVA analysis indicated that temperature had a highly significant effect on titres, with a very low P-value (3.95E-25), suggesting that temperature strongly impacts titre value of the vaccine. This finding aligns with a study by Soleimani and Rashid (2021) who reported that the storage of vaccine at high temperatures caused a decrease in potency and increased moisture content in the vaccine vials. On the other hand, the vaccine formulation has little and insignificant effect on the CBPP T1/44 vaccine titres. There was also no significant interaction between temperature and formulation, which means that these two are not interacting.



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Moreover, the One-Way ANOVA for pre and post lyophilization samples did not show any significant difference between formulations; thus suggesting that the lyophilization process did not affect the properties of the formulations significantly. The drying process does not introduce significant variations; hence, the formulations remain stable throughout. However, studies have proven that successful lyophilization can result in extended shelf lives and reduced cold-chain storage requirements, facilitating vaccine distribution to resource-poor areas (Preston and Randolph, 2021).

The formulation containing Casein plus Gelatin (Formulation 2) ranked best in this study, having demonstrated exceptional efficacy, maintaining high vaccine titres across various temperatures (4°C, 25°C, and 37°C) and throughout the study's time intervals (1, 3, 7, 10, and 14 days). This stabilizing agent showed better uptrend result in maintaining the titres than all the other stabilizers. The present outcomes are in agreement with the findings that revealed Casein and gelatin as one of the most effective stabilizers for vaccine production (Sangdjinan et al., 2022). According to Wahome et al. (2016), and Devarajan et al. (2022), gelatin provides thermostability and protection against freeze-thaw cycles, while casein helps maintain protein structure and integrity. Casein combined with gelatin might have formed a protective matrix that reduces moisture, prevent antigen degradation, and support effective lyophilization, which is crucial for long term stability. However, gelatin in vaccines has been linked to systemic allergic reactions, including anaphylactic shock (Sakaguchi and Inouye, 2000).

Also, it was generally observed that Formulation 2, 5 and 7 (all containing gelatin), showed an apparent increase in titre after freeze drying compared to their pre-lyophilization values. This trend was consistently noted across repetitions, although the magnitude of increase varied slightly between replicates. Among these, only two formulation number 2 continued to display unusually stable titres at 37°C, whereas the other gelatin-containing formulations followed the expected decline pattern. The Formulation (3) containing 16% skimmed milk used in CBPP vaccine production was the second best stabilizer in both 4°C and 25°C of storage but not in 37°C. This finding corroborates the report of El-Dakhly et al. (2019), which indicated the role of 10% skimmed milk in the stabilization of fowl pox vaccine (FPV) and pigeon pox vaccine (PPV). Similarly, Woodward and Tudor (1975) found an exceptional effect of using skimmed milk as a stabilizer in water vaccine for Newcastle disease (B1 type LaSota).

The result revealed that mycoplasma can be clearly well protected at 4°C and 25°C by skim milk + disaccharide sugar. However, the combination of skimmed milk, (disaccharide sugar) sucrose, or maltose and gelatin in vaccine (Formulations 5 and 7) demonstrated poor protection, suggesting that this particular mixture do not provide adequate stabilization or preservation for antigenic components. Previous studies have also reported similar findings in agreement to the roles of the disaccharide sugars, sucrose, maltose and skimmed milk as cryoprotectants in survival of *Lactobacillus rhamnosus* GG following one week storage at room temperature (Ghazy et al., 2017).

## Limit of the study

While this research explored the combined effects of components like skimmed milk, sucrose, or gelatin as stabilizers of the CBPP T1/44 vaccine, it did not investigate the precise mechanism by which they may enhance the vaccine stability and shelf life. Additionally, the study was conducted in a controlled laboratory environment, which may not fully replicate field conditions where temperature fluctuations can influence the vaccine stability.

# *Conclusion*

This study evaluated the performance of various stabilizers in enhancing the thermotolerance and shelf life of the CBPP T1/44 vaccine under conditions simulating those in sub-Saharan Africa. Formulation number 2 which contained Casein plus Gelatin demonstrated exceptional efficacy, maintaining high vaccine titres across various temperatures (4°C, 25°C, and 37°C) and throughout the study's time intervals (1, 3, 7, 10, and 14 days). Other formulations, such as Formulation 1 (Lactalbumin + Gelatin), Formulation 3 (Skimmed Milk), Formulation 4 (Skimmed Milk + Maltose), and Formulation 6 (Skimmed Milk + Sucrose), also showed promising results, maintaining decent viability at room temperature (20-25°C) for up to 14 days, though titres did not meet the WOAH requirements. The effect of high temperature (37°C) caused a significant reduction in vaccine viability, with Skimmed Milk + Sucrose + Gelatin offering the least protection at 37°C.

Based on the findings, vaccine manufacturers should use Formulation 2 (Casein plus Gelatin) as a stabilizer due to its high titre maintenance across various temperatures, with further studies needed to assess its efficacy in cattle. Additionally, Skimmed Milk alone or combined with disaccharides like Maltose or Sucrose can serve as alternative stabilizers, while further research is required to explore ways to reduce mycoplasma degradation and understand the



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titre fluctuations in certain formulations.

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## Ethical approval

Not applicable

## Conflict of interest

The authors do not have any conflict of interest.

## Author Contributions

The research concept was developed by Ally Omary Killo, Olayinka O. Ishola, and H.G. Ularamu. collected the samples. Laboratory analyses were conducted by Ally Omary Killo, Franklyn Ayomide Oluwadare and Foulematou Suma under the supervision of P.I Ankeli. Warsame Omar, Richard Rayson Sanga and Ally Omary Killo performed data analysis. The manuscript draft was prepared by Edmond Onidje and Ally Omary Killo and reviewed by Olayinka O. Ishola. All authors read and have given their approval for the publication of this article.

## Data availability

The data is available from the corresponding author, Ally Omary Killo, upon reasonable request.

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