Extension of Shelf Life of Raw Milk in Transit by Use of Lactoferrin and Lysozyme

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***Abstract*- The aim of the study was to extend the shelf life of raw milk in transit by use of lysozyme and lactoferrin. The effects of temperature, lysozyme concentration and lactoferrin concentration on milk in transit were evaluated by measuring pH, titratable acidity and methylene blue reduction time for eight hours. From the screening tests the Pareto plots indicated lysozyme concentration and lactoferrin concentration as factors of high influence. Further experiments were carried out focusing on the two factors to determine the optimum concentrations of lysozyme and lactoferrin to maintain a constant pH and titratable acidity for 8hours. A 3D Response Surface Methodology (RSM) was used to graphically map and optimise conditions. Optimum conditions were found to be 0.5g/l and 4g/l of lactoferrin and lysozyme and lactoferrin respectively, to maintain pH at 6.7 – 6.9, titratable acidity at 0.12 and methylene blue reduction time at 10.25 hours.615minutes.**

***Key words* – Lactoferrin, Lysozyme, Shelf-life, Milk, Optimisation**

I. INTRODUCTION

The collection of milk from farmers and transportation to the dairy is the most critical link in the total handling chain of milk. The most common method used to stop or retard the deterioration of milk on its way from the farmer to the dairy is cooling [1]. For most developing countries, this is not feasible because of the non-existence of cold chainsdue to lack of capital, lack of electricity, high operational costs and difficulties in the repair of equipment in rural areas. Prevailing high ambient temperatures often further compound the problem of milk collection in these areas causing a considerable loss of fresh milk. Thus, only part of raw milk reaches the dairy industry in anacceptable condition for use. In small scale dairy farms in the rural and peri-urban areas, post-harvest loses of milk are high due to microbial spoilage in transit caused by the afore mentioned reasons [1].

Milk has a number of inherent antimicrobial agents, these include lactoperoxidase, lactoferrin, lysozyme, xanthine oxidase, bovine immunoglobulins and bacteriocins, and probiotics[1], [2], [3]. Studies have shown that the strong microbial inhibitory effects of camel and goat milk arise from high concentrations of antimicrobial agents such as lysozyme and lactoferrin [4], [5], [6],hence the need to explore the use of these proteins in the preservation of raw bovine milk in transit. This research focused on the use of lactoferrin and lysozyme as a means to preserve raw cows` milk in transit. Lactoferrin and lysozyme work together for the stalling and destruction of some microorganisms. Lactoferrin and lysozyme are naturally found in milk but are in very low quantities [7]. As such, these should be added into the milk to achieve the bactericidal and bacteriostatic effectsneeded to extend the shelf life of raw milk at ambient temperatures. Both lactoferrin and lysozyme are non-specific to microorganisms, therefore, can target a large number of microorganisms [2], [8].

As most microorganisms need iron for growth, lactoferrin has the potential to inhibit microbial growth and even kill them by iron deprivation. The effectiveness of the antibacterial activity of lactoferrin depends on the iron requirement of the organism, the availability of exogenous iron, and the concentration and degree of iron-saturation of lactoferrin. It has been shown that 'natural' lactoferrin is bacteriostatic against a wide range of micro-organisms, including gram-negative bacteria with high iron requirements (coliforms, which are major mastitis pathogens), and also against some gram-positive organisms such as Bacillus species, and *Listeria monocytogenes* [3], [8]. Milk also contains *c* and *g* lysozymes. Lysozymes kill bacteria by disrupting the glycosidic bond between the two components of peptidoglycan, a constituent of the bacterial cell wall [4]. The enzyme usually functions in association with lactoferrin.

It is from this background that this research work sought to extend the shelf life of raw bovine milk in transit using lactoferrin and lysozyme. The objectives were to determine the effectiveness of the use of lysozyme and lactoferrin at different temperatures in raw milk preservation; to determine the shelf life of raw milk after the addition of lactoferrin and lysozyme, and to determine the optimum concentrations of lactoferrin and lysozyme using Response Surface Methodology (RSM). Through the knowledge of the preservative mechanisms of lactoferrin and lysozyme on raw milk, and how long they can extend he shelf life of raw milk in transit, it would aid in the quality maintenance of the milk, and preservation prior to processing.

II. MATERIALS AND METHODS

2.1 Milk Preparation

Milk used in the study was obtained from a dairy farm in the outskirts of Harare. The milk used for this experiment was obtained from the morning milking and stored for not more than 2 hours before collection. The milk was transferred in sterilised bottles to the laboratory.

2.2 Procedure for lysozyme and lactoferrin addition

The collected milk samples were divided into one-litre portions in the sterilised bottles. On arrival in the laboratory, chemical and microbial analysis were carried out on the milk. After which different lysozyme and lactoferrin concentrations were added to the milk.The samples were incubated at 25^{o}C and at 35^{o}C for 8 hours.

2.3 Effect of lysozyme and lactoferrin addition on milk pH

The initial pH of the milk on arrival to the laboratory was recorded. The samples with the different protein concentrations were incubated for 8 hours at 25^{o}C and at 35^{o}C and pH changes were noted at 2-hour intervals. The pH values were measured by direct insertion of a pH meter electrode (610, HANAH) into the milk sample.

2.4 Effect of lysozyme and lactoferrin addition on milk titratable acidity

The initial titratable acidity of the milk on arrival to the laboratory was recorded. For the different samples, titratable acidity was measured at 2-hour intervals. Titratable acidity as a percentage of lactic acid was measured [9].

2.5 Effect of lysozyme and lactoferrin addition on methylene blue reduction time

The initial methylene blue reduction time was recorded. For the different samples treated with the protein's methylene blue reduction, time was recorded at 2-hour intervals. One ml of the standard solution of methylene blue was added to 10 ml of milk sample. The tube was closed with a sterile rubber stopper, inserted with sterile forceps. It was then inverted slowly one or twice and placed in a thermostatically controlled covered water bath with a rack to hold the tubes immersed in the water at 37°C. The tubes were examined every half an hour and decolourisation were considered complete the when the column of milk was decoloured to 5 ml of the surface, the time of complete decolourisation was recorded [10].

2.6 Experimental Design

A two-level (2^{k}) full factorial was used to design the experiments where 'k' was the number of factors considered. In this instance, k was equal to 3, therefore, a minimum of 8 experiments were conducted (Table I). Coded values of (-1) low and (+1) high level for each factor were used. Factors considered for the experiments are the concentration of lactoferrin, concentration of lysozyme and temperature (Table II).

Table I: Treatment levels for the factors to be investigated (Generated by R Studio 1).12

Standard Experiment Order	Actual Experiment Order	Concentration of Lactoferrin (A)	Concentration of Lysozyme (B)	Temperature (C)\
1	3	-1	-1	-1
2	4	+1	-1	-1
3	1	-1	+1	-1
4	7	+1	+1	-1
5	2	-1	-1	+1
6	5	+1	-1	+1
7	8	-1	+1	+1
8	6	+1	+1	+1

2.7 Conversion of coded values and real time values

$$Real\ value = coded\ value \times 0.5(range) + centre\ value \quad \text{(Equation 1)}$$

$$Coded\ value = \frac{real\ value - center\ value}{0.5(range)} \quad \text{(Equation 2)}$$

$$Range = Maximum\ value - Minimum\ value \quad \text{(Equation 3)}$$

Table II: Coded values and Real time values used in the study

Factor	Coded Value	Real Value
.Lysozyme concentration (g/L)	-1	1
	+1	4
Lactoferrin concentration (g/L)	-1	0.4
	+1	1
Temperature (^{o}C)	-1	25
	+1	35

2.8 Screening of factors

The Pareto plot was used to screen factors and combinations of factors that have a significant effect on the storage time of raw milk.

2.9 Optimisation of results

Response surface methodology was used to optimise the effects of enzyme addition on milk pH, titratable acidity and methylene blue reduction time. Mathematical models obtained were validated by running additional experiments and the models were corrected to give optimum results.

III. RESULTS AND DISCUSSION

3.1 Effect of temperature, lysozyme and lactoferrin concentration on milk pH

It was observed that the pH of fresh milk was 6.7 to 6.9. The pH of the raw milk was used as an indicator of the activity of lactic acid producing bacteria which spoil the milk[11]. The effect of lactoferrin concentration, lysozyme concentration, and temperature on the final pH of the raw milk in transit was analysed using 3-D RSM. A 3FI model (Equation (4)) was generated for the final pH of milk ($\rho < 0.05$), and the corresponding coefficient of determination (R^2) was 0.9822. Equation (4) represented the response surface model fitted to the final pH of the treated milk:

$$pH = 6.185 + 0.1875A + 0.345B - 0.08C - 0.\mathbf{0575AB} + 0.0225AC + 0.031BC + 0.00125ABC \quad \text{(Equation 4)}$$

As shown in Table III, lactoferrin concentration and lysozyme concentration had a significant effect on the final pH of the milk whilst temperature of the milk had no significant effect on the final pH of the milk being preserved in transit. An increase in lactoferrin concentration and lysozyme concentration resulted in the maintenance of the initial pH of which ranged between 6.7 and 6.9. This could be attributed to the reduction in the number of viable spoilage microorganisms that would otherwise lower the pH of milk owing to fermentation due to the effects of lactoferrin and lysozyme.The synergistic interaction of lactoferrin and lysozyme has been shown to inhibit the growth of spoilage microorganisms through their bactericidal and bacteriostatic effects [12, 13]. When the pH value of the milk falls below 6.7, it typically indicates spoilage by bacterial degradation, mostly lactic acid bacteria (LAB) [11]. This was also supported by [17], who noted that higher LAB content corresponded with a decrease in pH, which can be attributed to the LAB acidifying capacity.

An increase in the temperature of the milk from 25^{O}C to 35^{O}C at a lactoferrin concentration of 0.4 g/L and lysozyme concentration of 4 g/L resulted in a decrease in the pH value of the milk from 6.7to 6.4. The reduction in pH could have been due to the increase in solubility of calcium phosphate and more calcium phosphate precipitates, releasing protons-H^+ with a gradual decline in pH with time [11].

The change in pH of milk might be attributed to changes in two salt systems whereraw milk contains 200mg CO_2 and about 50% of this is liberated on standing, with additional losses incurred on temperature increase due to heating. The net effect is a decrease in the titratable acidity and an increase in pH [14]. Colloidal calcium phosphate formation has a compensatory effect for the loss of CO_2mentioned earlier[15], [16].Most Gram-negativebacteria are resistant to lysozymes due to the presence of an outer membrane exterior to thepeptidoglycan, which protects the peptidoglycan for hydrolysis. This implies that the antimicrobial activity of Lysozyme is reduced [17]. This can explain the lowering of pH when lower concentrations of lysozyme and lactoferrin were used to treat the raw milk samples. This was observed when the concentration of lactoferrin was decreased from 1 g/L to 0.4 g/L at 25 ^{O}C, using lysozyme concentration of 1 g/L. From these pH results, it was observed that lactoferrin works symbiotically with lysozyme, where lactoferrin inhibits the growth of Gram-negative bacteria, and lysozyme inhibits the growth of Gram-positive bacteria[18].

Table III: Analysis of variance for the selected models.

Variables	Intercept	x_1	x_2	x_3	x_1x_2	x_1x_3	x_2x_3	$x_1x_2x_3$
Final pH	6.185	0.1875	0.345	-0.08	-0.05 75	0.0225	0.031	0.00125
p-values	-	0.0108	0.0014	0.1743	0.09	0.091	0.9666	-
F-values	-	32.31	130.26	3.14	3.2	3.24	0.0021	-
Titratable acidity	0.18125	-0.02 125	-0.04 625	0.00 875	0.00 625	-0.00 375	-0.00 125	-
p-values	-	0.0033	0.0001	0.1481	0.08	0.0698	-	-
F-values	-	91.20	231.20	3.20	3.6	4.12	-	-
Methylene blue reduction time	488.75	57.25	111	-1.25	-23	17.75	-13.5	-9
p-values	-	0.0259	0.0041	0.9339	0.1962	0.475	0.4758	-
F-values	-	17	63.92	0.0081	2.74	0.55	0.5	-

x_1, x_2, and x_3 are the main effects of lactoferrin concentration, lysozyme concentration, and temperature of the milk as independent variables. x_1x_2, x_1x_3, x_2x_3, and $x_1x_2x_3$ represent the interaction between lactoferrin concentration and lysozyme concentration, lactoferrin concentration and temperature of the milk, lysozyme concentration and temperature of the milk, and lactoferrin concentration, lysozyme concentration and temperature of the milk respectively. $\rho < 0.05$ is significant.

3.2 Effect of temperature, lysozyme and lactoferrin concentration on milk titratable acidity

Titratable acidity is important as it indicates the quality of raw milk and provides an indirect measure of the acid within the milk. Titratable acidity has been used to indicate whether the raw milk has undergone bacterial degradation or spoilage which is indicated by acid production [11]. It was observed that an increase in the concentration of lactoferrin concentration resulted in a corresponding decrease in the value of the titratable acidity of the raw milk. The effect of lactoferrin concentration, lysozyme concentration, and

temperature on the titratable acidity of the raw milk was analysed using 3-D RSM.

A 3FI model (Equation (5)) was proposed for the final pH of milk ($\rho < 0.05$), and the corresponding coefficient of determination (R^2) was 0.9856. equation presents the response surface model fitted to the final pH of the treated milk:

$$Titratable\ acidity = 0.18125 - 0.02125A - 0.04625B + 0.00875C + 0.00625AB - 0.00375AC - 0.00375BC - 0.00125ABC \quad \text{(Equation 5)}$$

where, A and B are lactoferrin concentration, and lysozyme concentration respectively.

As shown, in Table III, lactoferrin concentration and lysozyme concentration had a significant effect ($\rho < 0.05$), on the titratable acidity of fresh milk. However, it was noted that the temperature of the milk had no significant effect on the titratable acidity of the milk. An increase in the concentration of lactoferrin concentration from 0.4 g/L to 0.7 g/L, and 1 g/L at 1g/L lysozyme concentration and temperature of 25^{O}C for a storage time of 8 hours resulted in a decrease of the titratable acidity from 0.24 to 0.215, and 0.19 respectively. This could be attributable to an increase in the catalysing power of enzymes, as the rate of reaction increases with an increase in enzyme concentration. As a result, the bacteriostatic activity of lactoferrin is enhanced, thereby affecting more spoilage microorganisms within the milk. [19]. Lactoferrin decreases titratable acidity by sequestering the environment where some spoilage bacteria secrete their chelators to enhance iron uptake which is essential for their growth [2], [20].

An increase in the concentration of lysozyme resulted in a corresponding decrease of the titratable acidity of the milk. It was observed that when the lysozyme concentration increased from 1 g/L to 2.5 g/L, and 4 g/L at a lactoferrin concentration of 1 g/L and a temperature of 25^{O}C for 8 hours, there was a decrease in the titratable acidity from 0.19 to 0.14 and 0.12. This decrease in titratable acidity was because an increase in the concentration of the enzyme increases the rate of catalysis in the hydrolysis of glycosidic linkages in peptidoglycan, the structural polymer responsible for the strength and rigidity of the bacterial cell wall. This leads to the destruction or weakening of this layer which causes the cell to rupture or lyse
under osmotic pressure. Lysozyme is most active against Gram-positive bacteria, where the peptidoglycan is more readily accessible. As a result of these bactericidal mechanisms and effects, more Gram-negative spoilage bacteria are destroyed which results in the maintenance of titratable acidity. A lower titratable acidity can also be attributed to low concentrations of proteins and/or other buffering constituents within the fresh milk [21], [24].

A decrease in temperature resulted in a decrease in the titratable acidity of the milk and vice versa.

At lower temperatures of about 25^{O}C, the use of higher concentrations of lactoferrin and lysozyme decreases the titratable acidity of the milk to acceptable levels of 0.12 to 0.16. This is because lower temperatures will suppress the growth of LAB, and coliforms which require an optimum growth temperature of 37^{O}C [21]. In addition, lactoferrin and lysozyme will have bacteriostatic and bactericidal effects respectively on both the spoilage and pathogenic microorganism within the milk. This indicates that the milk will be preserved or maintain its quality if it is treated with higher concentrations of the enzymes lactoferrin and lysozyme with the milk being kept at temperatures between 25^{O}C to 30^{O}C.

3.3 Effect of temperature, lysozyme and lactoferrin concentration on methylene blue reduction time

Methylene blue reduction time determination is of importance as it determines the microbial load of the sample, that is it gives an idea of the microorganisms which will be utilising oxygen to produce lactic acid from lactose in the raw milk [11], [21]. The effect of lactoferrin concentration, lysozyme concentration, and temperature on the methylene blue reduction time of the raw milk was analysed using 3-D RSM.

A 2FI model (Equation (6)) was proposed for the final pH of milk ($\rho < 0.05$), and the corresponding coefficient of determination (R^2) was 0.9654. equation presents the response surface model fitted to the final pH of the treated milk:

$$Methylene\ blue\ reduction\ time = 488.75 + 57.25A + 111B - 1.25C - 23AB + 17.75AC - 13.5BC - 9ABC$$
(equation 6)

Itwas notedthat lactoferrin concentration and lysozyme concentration had a positive effect on the methylene blue reduction time of the raw milk. As the concentration of lactoferrin and lysozyme increased, the time required to reduce methylene blue also increased. It was observed that lysozyme concentration had a greater positive effect on the methylene blue reduction time compared to lactoferrin concentration. As shown in Table 3.1, lactoferrin concentration and lysozyme concentration had a significant effect on methylene blue reduction time. However, from the observed results, the temperature of the milk had no significant effect on the methylene blue reduction time of the milk.

Lactoferrin works as an antimicrobial compound through chelating the iron ion, making this essential ion unavailable to the invading pathogens. Lactoferrin affects the growth and proliferation of a variety of infectious agents, including both Gram-positive and Gram-negative bacteria, viruses, protozoa, or fungi [22]. Its ability to bind free iron, which is one of the elements essential for the growth of bacteria, is responsible for its bacteriostatic effect[23]. In addition, it has been shown that there are highly cationic areas on the surface of lactoferrin, which exert potent bactericidal effect by

interacting with elements of the bacterial membrane which are negatively charged, for example, lipotechoic and teichoic acids of Gram-positive bacteria, and lipopolysaccharides of Gram-negative bacteria [24], [25].

An increase in the concentration of lysozyme from 1 g/L to 4 g/L at a lactoferrin concentration of l g/L and a temperature of 25 ^{O}C, resulted in an increase from 419 minutes to 640 minutes. Microbial growth results in the oxygen depletion from milk and the formation of reducing substances during bacterial metabolism which cause the colour to disappear [26]. A higher methylene blue reduction time implies that the microbial load of the milk is low and the milk, is of good quality[11].

Acid fermentation of bacteria is common under ordinary conditions which are used by small scale farmers during the transportation of the milk from the milking places to the processing halls. At temperature from 10 to 37 ^{O}C souring is mainly due to *Streptococcus lactis*, *Enterococci*, *Lactobacilli*, and other coliform bacteria. The major psychrotrophic bacteria that have an impact on quality are the *Pseudomonas* species, while other species such as *Bacillus*, *Staphylococci*, and coliforms also have an effect [21]. As a result, an increase in temperature results in a decrease in the methylene blue reduction time, as indicated by the decrease in methylene blue reduction time from 640 minutes to 628 minutes when the temperature was increased from 25 ^{O}C to 35 ^{O}C at lactoferrin and lysozyme concentrations of 1 g/L and 4 g/L respectively after an eight-hour incubation period. This indicated that an increase in temperature will offer favourable and optimum conditions for the growth of spoilage microorganisms like *Streptococcus lactis*, *Enterococci*, and*Lactobacilli* which utilise oxygen during their growth and produce reducing compounds that will reduce methylene blue dye at a faster rate [21].

3.4 Optimisation of factors to maintain the quality of fresh raw milk in transit.

3.4.1 Final pH of treated raw milk

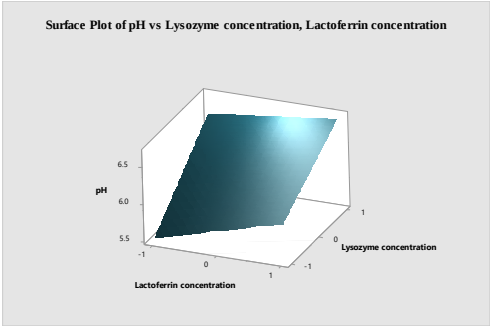

Figure 3.1: Response surface plot of the effect of lactoferrin concentration and lysozyme concentration on the pH of raw milk (Generated from Minitab 18).

3.4.2 Titratable acidity of treated raw milk

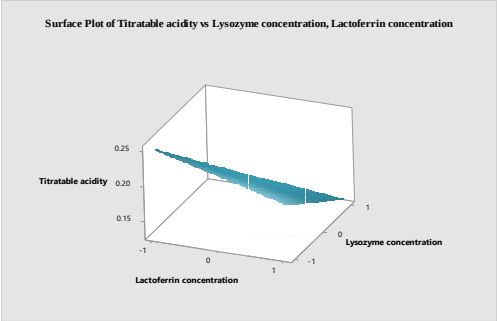

Figure 3.2: Response surface plot of the effect of lactoferrin concentration and lysozyme concentration on titratable acidity of raw milk (Generated from Minitab 18).

3.4.3 Methylene blue reduction time of raw treated milk

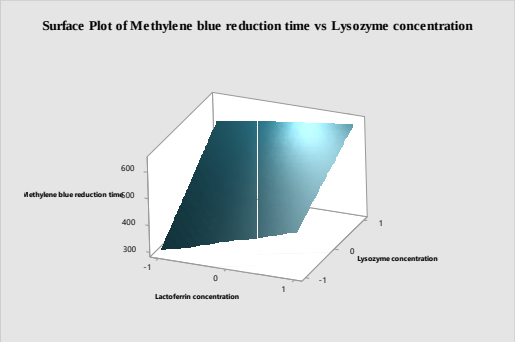

Figure 3.3: Response surface plot of the effect of lactoferrin concentration and lysozyme concentration on the methylene blue reduction time of raw milk (Generated by Minitab 18).

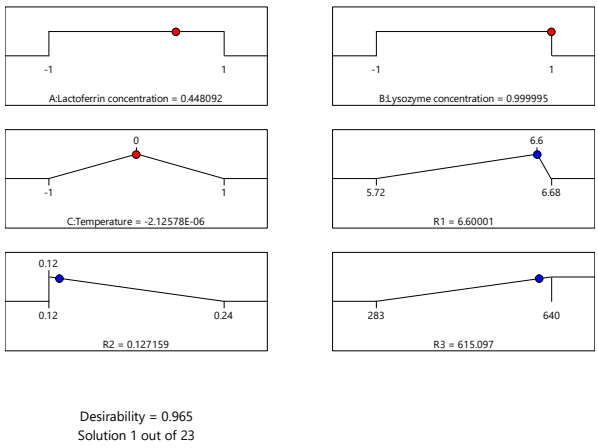

Figure 3.4: Ramp graphs showing the optimum conditions and expected outcomes (Generated from Design Expert 11).

3.4.4 The results obtained from the analyses of contour plots and response surface (Figure3.1, figure 3.2 and figure 3.3) and the ramp graphs (figure 3.4)showed that the optimum concentrations of both lactoferrin and lysozyme are approximately 0.5 g/L and 4 g/L respectively. These concentrations will ensure that the pH, titratable acidity, and methylene blue reduction time of the milk after 8 hours

without refrigeration will be approximately 6.7, 0.13, and 615 minutes respectively.

IV. CONCLUSION

Lysozyme and lactoferrin were effective in the preservation of raw milk at temperatures between $25^{O}C$ -$35^{O}C$.

The shelf life of raw milk after the addition of lysozyme and lactoferrin at optimum conditions was found to be 10.25hours, which was greater than the 8 hours which was targeted at the beginning of the study. Response surface methodology was used to determine optimum conditions after screening temperature as it was found to have minimal effect after addition of lysozyme and lactoferrin. The optimum concentration for lactoferrin and lysozyme were found to be 0.5g/l and 4g/l respectively

The use of natural methods of milk preservation may help reduce the milk loses due to lack of refrigeration and as well unreliable refrigeration systems [22].

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