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Research Article

The Effect of Combined Clove and Cinnamon extracts on Growth and Survival of *Escherichia fergusonii* and *Salmonella typhimurium* in milk pre and post fermentation

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Abstract

The antimicrobial activities of extracts of clove buds (CL) and cinnamon bark (CE) were investigated individually and in combination in fermenting and fermented full cream milk against *Escherichia fergusonii* and *Salmonella typhimurium*. Clove and cinnamon were extracted for their essential oils (EOs) and eugenol and cinnamaldehyde were the major components representing 60-80% of the total oils. Preheated milk was inoculated with 1% (v/v) of a mixed culture of *Lactobacillus delbrueckii* subspecies *bulgaricus* (NCIMB 11778) and *Streptococcus thermophilus* (NCIMB 10387) and incubated at 25 or 37^{o}C for 24 h. *E. fergusonii* or *S. typhimurium* (3 x 10^5 CFU/mL) were introduced into the milk pre - or post-fermentation. CL and CE were added at the same time as the pathogens based on their Minimum

Inhibitory Concentrations of 0.25% for both pathogens as follows: CL and CE at 0.25% each alone; 0.0625% CL/0.1875% CE; 0.125% CL/ 0.125% CE; and 0.1875% CL/0.0625% CE. When added at the start of fermentation at 25°C, the CL and CE combinations inhibited the growth of *E. fergusonii*, and *S. typhimurium*, whereas at 37°C and associated with more rapid and higher acidification, the CL and CE combinations showed a marked antimicrobial activity against both pathogens. In post fermentation inoculated milk, survival of *E. fergusonii* and *S. typhimurium* was reduced by all CL and/ or CE treatments and were undetectable in samples containing 0.0625% CE with 0.1875% CL and 0.1875% CE with 0.0625% CL within 48 to 72 h of storage. The extent of the effect was most marked in the samples fermented at 37°C compared to 25°C and related to the pH after fermentation of 4.2 ±0.2 and 5.0 ±0.2 respectively. The use of these extracts both during fermentation and storage have the potential to enhance the microbiological safety of these products.

Key words: Natural antimicrobial; Clove extract; Cinnamon extract; *Escherichia fergusonii*; *Salmonella typhimurium*; Traditional fermented milk; Pre and post fermentation contamination

1. Introduction

Pastoralists are increasingly producing milk for the market as well as for subsistence, contributing to the growth of a production subsector with notable resilience to climate variability and change. Milk presents livelihood opportunities to most of the rural populations in Africa ranging from farmers, processors, shopkeepers, and other stakeholders in the dairy chain [1, 2]. Milk and milk products have excellent nutrients as milk consists of fats, proteins, minerals, vitamins, carbohydrates, and water [3]. Milk, however, is extremely susceptible to spoilage by microorganisms. The quality control of milk is an essential aspect of the dairy chain [4] to ensure milk product safety and public health. With lack of good quality water, unreliable electricity, and poor keeping quality of the milk, contamination of the milk is unavoidable even if the milk was pasteurised or under very hygienic conditions of milk production [4]. Contamination of milk can occur at any stage during handling from production side, storage to marketing between animal and consumers [5]. This number may increase considerably according to the type of bacteria, their virulence, and surrounding conditions especially temperature. Consumption of unpasteurised milk has led to many outbreaks and pasteurised milk has also been responsible for causing illness through post process contamination. Many Africans prefer sour, or acidified milk made from raw milk where there is no denaturing of milk proteins by heat treatment process, or destruction of natural flavours in the milk [6,7]. Rural African farmers incorporate some spices into fermenting/fermented milk either as whole (unbroken) spices or as coarse powders to improve the flavour and aroma of the product [8]. Cava et al. [9] reported the effect of essential oils of some spices on pathogens in milk. Furthermore, Ogwaro et al. [10] and Cava et al. [11] reported on the effect of fractional combinations of spices against various food pathogens. There are, however, no reports on the effects of combined essential oils of cloves with cinnamon on *Escherichia fergusonii* and *Salmonella typhimurium* in fermented milk. *Escherichia fergusonii*, is a Gram-negative, rod-shaped bacterium formerly known as, Enteric Group 10 due to its biochemically distinct nature compared to other species and bio-groups of Enterobacteriaceae. It is one of the five members of *Escherichia* which can be found in the intestines of human beings [12]. *E.*

fergusonii was isolated from raw milk and some dairy products in Egypt [13]. According to Sherwood and Clegg [14], *E. fergusonii* grows optimally at 37-40^{o}C under aerobic conditions but temperature range of growth extends from 21- 45^{o}C so the bacterium is well suited to growth in tropical African temperatures. However, its prevalence and survival in milk and milk products is not yet well documented. Salmonellae are Gram negative short rods bacteria of the family Enterobacteriaceae. They have caused outbreaks of illness around the world and continue to be a major concern for the dairy industry. They are widespread in the environment and appear in a wide variety of foods and food ingredients [15]. Salmonellae have been isolated in raw milk [16]. In principle, using raw milk increases the likelihood of Salmonellae being present in the product. Salmonella grow optimally between 35 to 37^{o}C, but they can grow at much lower temperatures too. The pH range for growth of Salmonellae is between 6.5 and 7.5 but it can also grow readily in an acidic environment [17]. Under laboratory condition, Salmonellae grew at pHs as low as 4.05, 4.10 and 4.40 in the presence of hydrochloric, acetic, and lactic acids respectively [18]. Yoghurt has been reported to be effective in controlling growth of Salmonella [19]. However, Adams and Hall [20] and Alvarez-Ordonez, [21] reported that inhibition of Salmonellae in the presence of lactic acid bacteria depended on the type of lactic acid bacteria, incubation temperature, and size of inoculum of lactic cultures. Clove, (*Syzygium aromaticum L. Myrtaceae*) also known as 'champion' spice is an aromatic plant widely cultivated in tropical and subtropical countries. It is one of the most intensely flavoured spices and extensively used in foods and hot drinks such as tea and many brands of tomato ketchup [22]. Clove oil is a mixture of different compounds with three main active ingredients and is rich in volatile compounds and antioxidants such as eugenol, β-caryophyllene, and α-humulene. Clove oil has biological activity relevant to human health including antimicrobial, antioxidant, and insecticidal, antifungal, and antiviral activity [23]. Antimicrobial activity of cloves has been reported by many researchers [11, 24]. Xu et al. [25] reported that clove could destroy the cell walls and membranes of microorganisms, and permeate the cytoplasmic membranes or enter the cells, then inhibit the normal synthesis of DNA and proteins. Eugenol accounts for at least 60-80% of the composition and thus is the main active component. The remaining 20-40% consists of eugenol acetate and β -caryophyllene and alpha-humulene [26]. Xu et al. [25] also noted that eugenol could inhibit the production of amylase and proteases in *Bacillus cereus* and Burt [27] reported that clove has the ability to deteriorate cell wall causing cell lysis. Cinnamon (*Cinnamomum zeylanicum*) on the other hand is from the Lauraceae family and is used widely in the preparation of tea and foods for its flavour and aroma. The three main components are cinnamaldehyde, cinnamyl acetate and cinnamyl alcohol. Cinnamon is reported to have antimicrobial compounds [28, 29] and it is active as an antibacterial, anti-allergic, anti-ulcerogenic, antipyretic and antioxidant [25]. Research has shown that cinnamon extract was more effective than cinnamon essential oil [11, 30, 31]. The aim of this study was to assess the antimicrobial activity of a mixture of clove and cinnamon extracts on the growth and survival of *E. fergusonii* and *S. typhimurium* in pasteurized full milk during and post fermentation

2. Materials and Methods

2.1 Media for growth

Tryptone Soy Broth (TSB, LAB004) Tryptone Soy Agar (TSA, LAB011); de Man-Rogosa-Sharpe Agar

(MRSA LAB098); de Man Rogosa-Sharpe Broth (MRSB LAB093) and M17 Agar (LAB092) were all purchased from Lab M Limited (Bury, UK) and M17 Broth (CM0817) was purchased from Thermo Fisher Scientific (Loughborough, UK). All the media were prepared according to the manufacturers' instructions. M17 Agar and M17 broth were used for *S. salivirus* subspecies *thermophilus* [32]. 50 mL of 10% sterilized lactose was added to 1 litre of M17A or M17B before use [33]. The sample was tested by streaking on the agar or inoculating in the broth using a typical *S. thermophilus* colony and incubated at 37°C for 24h. MRSA and MRSB were used for the enumeration of *L. delbrueckii* subspecies *bulgaricus* [34], Violet Red Bile Agar (VRBA, CM0107B, Thermo Fischer Scientific, Loughborough, UK) for the enumeration of E. fergusonii and Xylose Lysine Deoxycholate (XLD) (CM0469, Thermo- Fisher Scientific, Loughborough, UK) for *Salmonella typhimurium.* Sterile quarter-strength Ringers Solution (BR 0052, Thermo Fischer Scientific, Loughborough, UK) was used as an isotonic diluent for the bacterial cells. All media were prepared with deionized water.

2.2 Microorganisms and Culture Conditions

Escherichia fergusonii (UCC 585) was isolated and identified from a traditional African yoghurt. *S. typhimurium* (NCIMB 10248) *L. delbrueckii* subspecies *bulgaricus* (NCIMB 11778) and *Streptococcus thermophilus* (NCIMB 10387) were obtained from the National Collection of Industrial Food and Marine Bacteria (UK). Before each experiment, a culture from a freeze-dried vial, maintained at -20°C was activated in TSB for *S. typhimurium*; and in M17 Broth for *S. thermophilus* incubated for 24 h at 37°C ±0.1°C and streaked onto TSA, and M17 Agar respectively; and *L. delbrueckii* subspecies *bulgaricus* was activated in MRSB, pH 5.5 ±0.2 and incubated under anaerobic condition at 37 ±0.1°C for 48-72 h. The pH of MRS agar and broth were adjusted with 1N HCL to pH 5.5 ±0.2.

The cultures were then streaked on MRSA and incubated in anaerobic gas jar at 37°C for 48-72 h. Resuscitated microorganisms were sub-cultured twice before use in the experiments. All activated cultures were maintained on slants at 4ºC and were sub-cultured monthly. For the growth of S. *thermophilus,*M17 Agar or M17 Broth were used. 50 ml of 10% sterilized lactose was added to the M17 A or M17 B before use. The sample was tested streaking on the agar or inoculating in the broth using typical *S. thermophilus* culture incubated at 37°C for 24h. To prepare the inoculum, one pure isolated colony from a plate agar was transferred to TSB for *S. typhimurium* and *E. fergusonii*, M17B for *S. thermophilus* and MRSB (pH 5.5) for *L. delbrueckii* subspecies *bulgaricus.* The latter was incubated in anaerobic gas jar. All the cultures were incubated at 37°C for 24 h with shaking (150 rpm). *L. delbrueckii subspecies bulgaricus* grew better at pH 5.0-5.5 while *S. thermophilus* grew well at pH 6.0. The lactic acid bacteria were inoculated at approx. 10^6 CFU/mL.

To obtain the viable counts for *E. fergusonii* or *S. typhimurium* during the antimicrobial challenge test, serial dilutions were made in ¼ strength Ringer's solution and 100 µL was plated out on VBRA and brilliant green agar respectively incubated for 24 h at 37°C. Viable counts were recorded after the incubation period.

2.3 Spices for this study

Spices in this study were clove buds and cinnamon barks (Table 1), purchased from the local market in Juba, South Sudan

2.4 Extraction of essential oils

The spices were first washed with sterile distilled water then dried in a drying cabinet at 50°C for 72 h. The dried spices were then crushed individually using a sterilized mortar and then ground with an electric grinder to coarse smaller particles before extraction. Clove and cinnamon essential oils (EOs) were extracted using Soxhlet extractor according to the methods of Azwanida [35]. Briefly, 100 g of each ground powder was weighed in a 50 mL extraction thimble. This was placed inside the main chamber of the Soxhlet extractor. 500 mL of HPLC grade methanol (Sigma Aldrich, UK) was put in a 1000 mL round bottom flask and was placed on the electric heater of the extractor. The heater was turned on to 50°C (methanol's boiling point). Continuous extraction took place by re-fluxing the solvent until the solvent was clearly colourless. Using a rotary evaporator, the methanol was evaporated at fixed temperature of 50°C until all the methanol was completely evaporated off, leaving a thick essential oil. The samples were either used immediately or kept at -20°C until use (used within one month). The compositions of EOs from the spices were determined by Gas Chromatography-Mass Spectrometry (GC-MS).

Common name	Scientific name	Part of the plant	Extraction method
Cinnamon	*Cinnamomum zeylanicum*	cinnamon bark	Steam distillation
Clove	*Eugenia caryophyllata*	clove buds	Steam distillation

Table 1: Spices selected in this study and the oil extraction methods

2.5 Milk sample preparation

Whole, full cream pasteurized milk (pH 6.7 ±0.1) was purchased from a supermarket one day before the experiment was performed and at least 6 to 7 days before its use-by date. Sterile test tubes containing 10 mL of milk were heated by steam for 30 min at 85°C and transferred immediately to cool in a water bath set at 40°C. The sterility of the samples was confirmed by streaking on to petri dishes of TSA and incubating at 37°C for 4 h.

2.6 Pre-screening for antimicrobial activity

Antimicrobial activity was tested by agar-well diffusion method. 1000 µL of undiluted overnight inoculum of either *E. fergusonii* or *S. typhimurium* was inoculated in cooled (45^{o}C) but still molten 20 ± 2 mL TSA. After mixing, the agar was poured into the Petri dish in duplicates. Another 20 ml of molten agar (without the bacteria), in duplicate was poured in sterile petri dishes as control. This was allowed to set for 1-2 hours in the laminar flow (LAF) cabinet then four, equal distance holes were punched in each plate of the agar. To each well, 150 µL of the individual or combined spice extract was added and left to stand for about 1 hour to allow the extract to diffuse. The plates were then incubated for 24 hours at 37°C. The antimicrobial activity was evaluated by measuring the Zone of inhibition (ZOI) using a ruler and expressed in millimetres (mm).

2.7 Determination of Minimum Inhibitory Concentration (MIC)

MIC was determined by broth dilution methods. 1% (v/v) stock solution of each EO of spice extract was prepared in methanol (solvent). To determine the minimum inhibitory concentration of the EOs, 12 test tubes, each containing 5.0 mL of pasteurised milk were set up. From the 1.0% of spice extract prepared before, a 2-fold serial dilution was carried out in tubes 1-10 to dilute to 0.156%. The 11th and 12th

tubes contained pasteurised milk only. 100 µL of the suspended *E. fergusonii* or *S. typhimurium* was then added to test tubes 1-11 giving a final concentration of approx. 10^5 CFU/mL in each tube. Counts were initially performed to ensure that the size of the inoculum was in the range desired (x 10^5 CFU/mL). The control sample (Tube 11) contained *E. fergusonii* or *S. typhimurium* without the spice extracts. Tube 12 contained pasteurised milk only (sterility control). All the test tubes were then incubated at 37°C for 24h. Following the incubation, the samples were serially diluted (1:10) in quarter Ringer's solutions and appropriate dilutions were plated on TSA plates. The plates were incubated at 37°C for 24 h. The lowest concentration of the EO treatment that inhibited visible growth of the pathogen after incubation was taken as the MIC of the treatment. The minimum bactericidal concentration (MBC) was carried out by plating 100 µL from each tube was plated on TSA plates and the MBC was defined as the lowest concentration at which no growth was observed.

2.8 Effect of combined essential oils of cloves (CL) and cinnamon (CE) extracts on *E. fergusonii* and *S. typhimurium* during fermentation (pre-fermentation contamination)

The CL and CE were used alone or in combination with each other at ¼, ½ and ¾ MIC (Table 2) to determine the antimicrobial effectiveness against *E. fergusonii* or *S. typhimurium* when contamination occurs pre- fermentation or post fermentation. For the pre-fermentation contamination, milk samples were inoculated with 1% (*v/v, about* 10^6 *CFU/ mL*) of a mixed culture of *L. delbrueckii* subspecies *bulgaricus* and *S. thermophilus*. To assess the effect of the combined CL with CE on growth of the pathogens during fermentation, 100 µL of a diluted overnight culture of either *E. fergusonii* or *S. typhimurium* was added to all the 6 tubes to give a final concentration of approx. 10^5 CFU/mL was added to each sample along with the treatments of CL and CE either alone or in combinations as shown in table 2. Triple sets of the test tubes were then incubated for 24 h at 25°C or 37°C. Tube No. 1 was inoculated with the test bacterium but without the spice extracts. This was included in each experiment and served as control. Viable counts during fermentation were enumerated at 0, 2, 4, 6, 8, 10, 12 and 24 h. The cell counts were converted into Log_{10} CFU/mL and plotted against time.

Tube No.	CL conc. (%)	CE conc (%)	MIC of CL	MIC of CE
1	0	0	0	0
2	0.25	0	1	0
3	0.0625	0.1875	1/4	3/4
4	0.125	0.125	1/2	1/2
5	0.1875	0.0625	3/4	1/4
6	0	0.25	0	1

Table 2: Concentrations of CL and CE evaluated for antimicrobial effects against *E. fergusonii* and *S. typhimurium*.

2.9 Effect of combined essential oils of cloves extract (CL) and cinnamon extract (CE) on survival of *E. fergusonii* and *S. typhimurium* during storage (post-fermentation contamination)

For the study of post contamination effect of the extracts on the survival of the bacteria, initially a set of pasteurized full cream milk samples were inoculated with 1% (v/v) of mixed culture of lactic acid bacteria (*L. delbrueckii* subspecies *bulgaricus*

and *S. thermophilus*) only and were incubated at 25°C or 37°C for 24 h. After the fermentation, approx. 10^5 CFU/mL stationary phase cultures of *E. fergusonii* or *S. typhimurium* was added to each sample along with the combined treatments of CL and CE (Table 1). Each treatment was stored at 25°C for 120 h. Viable counts for *E. fergusonii* and *S. typhimurium* were determined by plating 100 µL of each sample on VRBA or XLD respectively at 0 [start of storage], 24, 48, 72,120, and 144 h during storage.

2.10 pH measurement

The pH of the samples was measured with a Mettler Toledo Delta 320 pH meter, at room temperature. Readings were taken before inoculation (negative control), immediately after inoculation (T=0) and then at every sampling point.

2.11 Measurement of titratable acidity

20 g of a well shaken yoghurt or un-fermented milk was weighed accurately into a 250-mL Elenmeyer flask, 40 mL of boiled and cooled distilled water was added to it. With a sterile pipette, 2-3 drops of phenolphthalein was added in the milk as an indicator of end point. The content of the flask was titrated against 0.1N sodium hydroxide (NaOH) until the sample changed colour to persistent light pink. The initial and final readings on the meniscus burette were recorded, prior to starting the titration and at the end point, respectively.

The amount (mL) of 0.1N NaOH titrated was calculated by subtracting the initial volume from the final volume to give the amount of NaOH used to reach the endpoint. This was performed at least three times per sample. The amount percent lactic acid was then calculated using the equation:

$$\text{Titratable acidity [\%]} = \frac{\text{Volume of titrant} \times N \times 90 \times 100}{\text{volume of sample} \times 1000}$$

Where N = normality of titrant; 90 = Equivalent weight for lactic acid

2.12 Statistical Analysis

All experiments were performed in triplicate. The data were expressed as the mean ± SD. The differences between means ($P<0.05$) were compared by one-way analysis of variance (ANOVA) using Prism graph pad (USA), version 9.0.

3.0. Results

3.1 Chemical composition of methanol clove buds and cinnamon bark extracts

The chemical composition of methanol clove buds and cinnamon barks were identified by Gas Chromatography- Mass Spectrometer (GC-MS). Quantitative calculations were based on the relative areas of the corresponding GC signals. Seven components were identified in clove bud's extract. Eugenol was the major component comprising of 68.76% followed by Caryophyllene then Phenol, 2-methoxy-4-(2-propenyl) acetate (Table 3). Seven compounds were identified in cinnamon barks extracts (Table 4). The major component was cinnamaldehyde comprising of 83.54% followed by alfa-Copaene and cis-Calamenene, alpha-Muurolene.

No.	Compound	Retention Time (min)	Relative content (%)
1	Methylamine, N,N-dimethyl-	1.51	8.28 ±0.3
2	Eugenol	11.44	68.77 ±0.3
3	Phenol, 2-methoxy-3-(2-propenyl)-	11.51	1.16 ±0.3
4	Caryophyllene	12.33	11.04 ±0.3
5	Humulene	12.76	1.44 ±0.3
6	Phenol, 2-methoxy-4-(2-propenyl)-, acetate	13.54	7.01 ±0.3
7	(4-Acetylphenyl)phenylmethane	15.52	2.30 ±0.3

Relative content values are mean ± standard deviation (n=3)

Table 3: The analysis of chemical composition of clove buds extracts as analysed by GC-MS.

No.	Compound	Retention Time (min)	Relative content (%)
1	Cinnamaldehyde, (E)-	10.23	83.46 ±0.25
2	alfa-Copaene	11.72	3.02 ±0.45
3	Coumarin	12.55	1.81 ±0.5
4	gamma-Muurolene	13.02	0.86 ±0.5
5	alpha-Muurolene	13.29	2.24 ±0.35
6	cis-Calamenene	13.58	2.84 ±0.35
7	2-Propenal, 3-(2-methoxyphenyl)-	13.63	5.76 ±0.35

Relative content values are mean ± standard deviation (n=3) ± SD

Table 4: The analysis of chemical composition of cinnamon extracts as analysed by GC-MS

3.2 Preliminary assessment of the antimicrobial activity of CL and CE by agar well diffusion

The initial screening of the antimicrobial activity of CL or CE against *E. fergusonii* and *S. typhimurium* was carried out using agar well diffusion. CL and CE extracts were assayed in TSA using six concentrations (1%; 0.5%; 0.25%; 0.125%; 0.0625% and 0.0312%). The zones of inhibition of the cloves or cinnamon extracts against *E. fergusonii* and *S. typhimurium* are presented on Table 5. At 1% (w/v) concentration of CL or CE, the zones of inhibition ranged from 16-18 mm and 14-15 ±0.5 mm for *E. fergusonii* or *S. typhimurium* respectively. At 0.5 and 0.25% (w/v), similar zones were observed, 8.9 ± 0.5 mm and 7-8 ±0.5 mm for both pathogens but at 0.125% (w/v), cloves extract showed no ZOI against *E. fergusonii*. At 0.0625%, no zone was detected for *E. fergusonii* nor *S. typhimurium*. In view of the observations in the agar well diffusion method, the MIC of the extracts was assessed with the broth dilution assay with concentration from 1 to 0.0156%. The MIC was found to be 0.25% and MBC was 1% for both clove and cinnamon extract for both *E. fergusonii* and *S. typhimurium*.

3.3 Effect of combined concentrations of CL and CE on the growth of *E. fergusonii* and *S. typhimurium* and change in pH of milk during fermentation at 25 or 37°C

***3.3.1* Growth of *E. fergusonii* in milk during fermentation at 25 or 37°C:** Figure 1A shows growth curves of *E. fergusonii* in the LAB fermenting milk with various concentrations of CL and CE at 25°C. *E. fergusonii* grew to a similar level in all the samples in the initial 4 h then, in the control and the sample with 0.25% CE alone, it increased from the initial 10^5 CFU/mL to 10^9 CFU/mL (doubling times 0.68 h). Growth of the bacterium in the samples treated with the combined concentrations of the EOs resulted in lower counts than the counts in the control samples. The most potent combination at the fermentation temperature of 25°C was 0.1875% CL

(three-quarters MIC) combined with 0.0625% CE (one-quarter MIC). Growth in this sample was slower (doubling time 1.5 h) than in the control sample (doubling time 0.68 h). At this concentration the cell counts were approx. 10^6 CFU/mL, 1 log unit lower than the initial contamination level and approximately 2.4 log units lower than the counts in the control samples.

The next most effective combination was the 0.125% CL (one half MIC) with 0.125% CE (one-half MIC). In the other treated samples, the bacterium grew to approx. 10^8 CFU/mL and $10^{8.5}$ CFU/mL respectively. At this temperature, the pH of the milk declined from initial pH 6.8 ±0.1 to 6.5 (±0.2) in all the treated samples after 4 h then to pH 5.3 (±0.2). The pH with 0.25% CL only was higher than in all the samples even in the control. This was in line with titratable acidity being lowest in that sample too. Statistical analysis showed that there was no significant difference ($p>0.05$) between the control and treated samples in the initial 8 hours at 25°C. After this time, there was a significantly less growth in the treated samples except CE alone. Figure 1B shows the growth pattern when the milk was fermented at 37°C in the absence or presence of combinations of CL and CE. In the absence of extracts and in the sample with 0.25% CE alone, the bacterium grew to approx. 10^9 CFU/mL in the first 12 h of incubation (doubling time 0.56 h and 0.41 h respectively) and subsequently in the control (samples without treatment), it increased by approx. 1 log unit more than in the sample incorporated with combined CL 0.25% alone.

However, in the sample with 0.25% CE alone the increase was less as seen in figure 1B. Although CE alone was not statistically significantly different to the control, all other CE/CL treatments significantly reduced the E.fergusonii population ($p<0.05$) and 0.1875% CL/ 0.0625% CE to undetectable levels in 6 h.

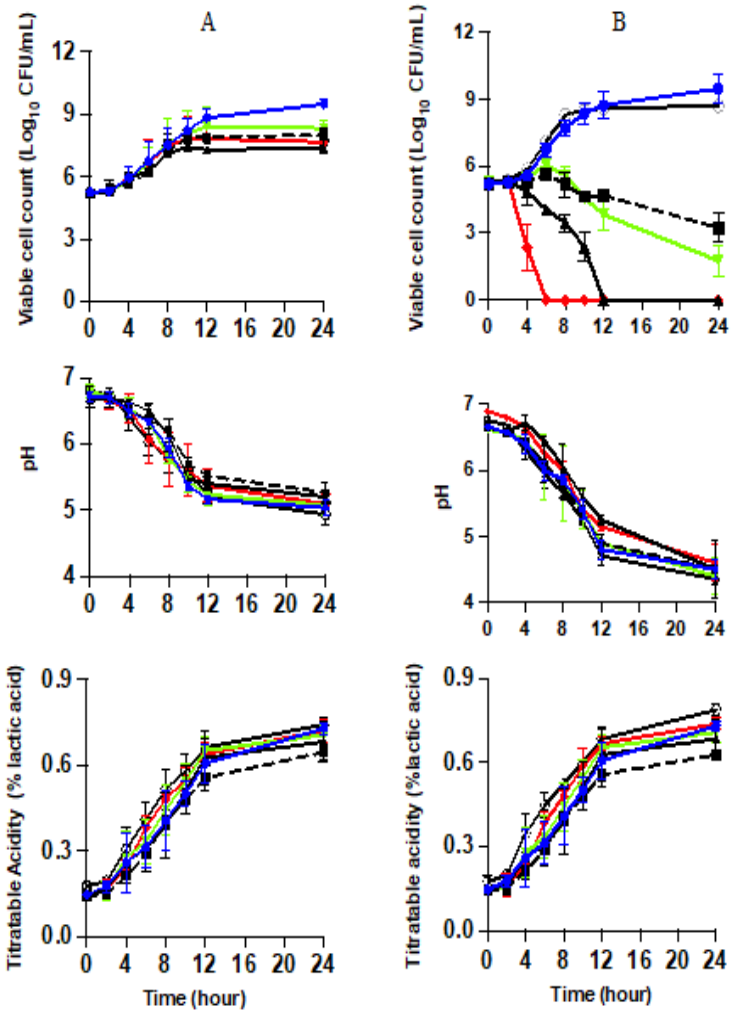

Figure 1: Growth of *E. fergusonii*, change in pH and titrateable acidity in fermenting milk in the presence of clove (CL) and cinnamon (CE) extracts at different concentrations at A) 25°C and B) 37°C for 24 h. Values are the mean of three individual replicates (means ± SD)

Control [●]; 0.25% CL alone [--■--]; 0.0625% CL / 0.1875% CE [-▲-]; 0.125% CL / 0.125% CE [▼]; 0.1875% CL / 0.0625% CE [◆]; 0.25% CE alone [-o-].

***3.3.2* Growth of *S. typhimurium* in milk during fermentation at 25 or 37°C:** The growth curves of S. *typhimurium* milk fermenting at 25°C are shown in Figure 2A. S. *typhimurium* showed a similar growth pattern in response to all treatments in the first 4 h. In the control sample, the bacterium then increased from 10^5 CFU/mL to 10^8 CFU/mL (doubling time 0.86 min) after 10 h then to 10^9 CFU/mL after 24 h fermentation. Whereas the samples treated with combined extracts: 0.0625% CL / 0.1875% CE; 0.1875% CL / 0.0625% CE and the 0.125% CL / 0.125% CE, at 24 h the viable cell counts were 10^6 CFU/mL; $10^{6.5}$ CFU/mL and 10^7 CFU/mL respectively with doubling times of 1.5 h. The pH declined in all the samples and was lowest in samples containing 0.25% CE only. It declined from pH 6.8 (± 0.1) to pH p.5 (± 0.1) after 12 h of fermentation. The highest pH was in the sample containing combined 0.125% CL with 0.125% CE with pH 5.1(± 0.1) after 24 h. The TA of the samples was in the

range of 0.61-0.7% lactic acid in all the samples (Figure 2A). Growth curves of *S. typhimurium* at milk fermented at 37°C are presented in figure 2B. The results showed that growth was inhibited in all the treated samples except for the sample with 0.2% CE% alone which was approx. 1 log unit lower that the control sample. In samples treated with 0.1875% CL with 0.0625% CE, the *S.typhimurium* population was reduced as soon as the fermentation commenced and was not detected after 6 h of fermentation. In samples treated with 0.0625% CL / 0.1875% CL (one quarter MIC of CL with three quarter MIC of CE) and containing 0.125% CL /125%CE (one half with one half), cell numbers declined to below 10^3 CFU/mL and 10^3 CFU/mL respectively. The acidity of the samples increased with the pH around 4.5 and TA ranged between 0.7% - 0.8% lactic in sample with 0.25% CL. The pH in all the samples declined similarly up to 12 hours and this was reflected by an increase in titratable acidity over this period. This shows that lactic fermentation had progressed in the presence of CE/CL but the addition of the extracts contributed to the reduction in the pathogen levels. With continued fermentation, the pH declined in all the samples to pH 4.1–4.6 (±0.2) after 24 h.

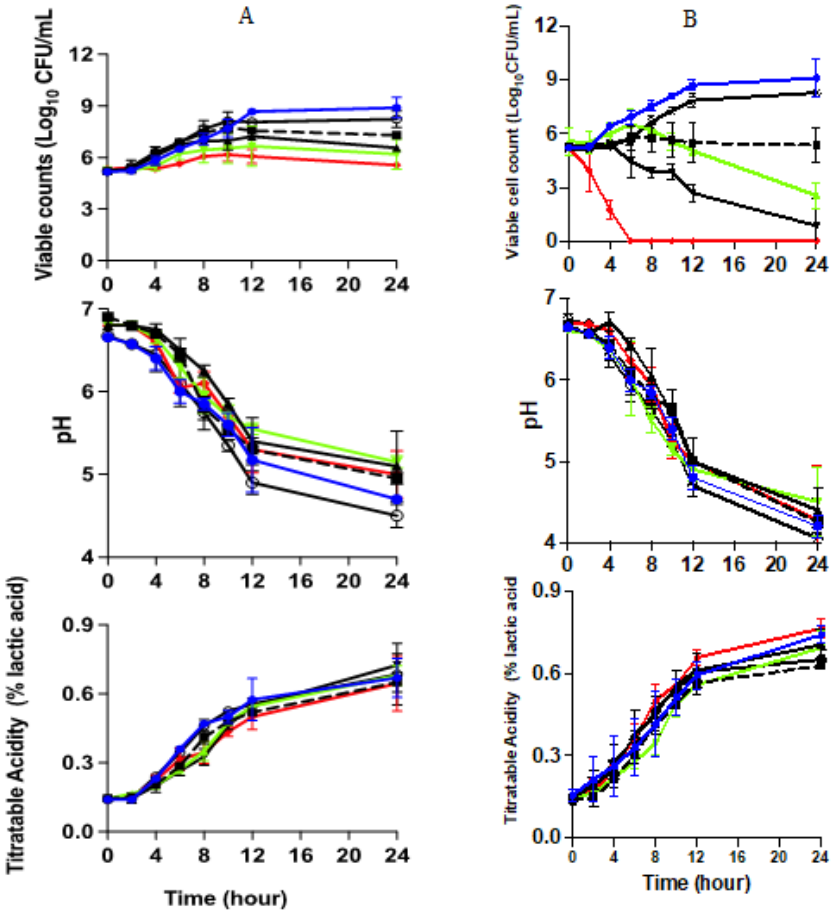

Figure 2: Growth of *S. typhimurium*, change in pH and titratable acidity in fermenting milk in the presence of clove (CL) and cinnamon (CE) extracts at different concentrations at A) 25°C and B) 37°C for 24 h. Concentrations are in % (w/v), values are the mean of three individual replicates (means ± SD). Control [●]; 0.25% CL alone [--■]; 0.0625% CL / 0.1875% CE [-▲-]; 0.125% CL / 0.125% CE [▼]; 0.1875% CL / 0.0625% CE [◆]; 0.25% CE alone [-o-].

3.4 Effect of combined concentrations of CL and CE on the survival of *E. fergusonii* and *S. typhimurium* and change in pH post fermentation

3.4.1 Survival of *E. fergusonii* during storage in milk fermented at 25°C or 37°C and stored at 25°C: Milk was fermented at 25°C for 24 h thereafter, various concentrations of CL mixed with CE were added to it as well as 10^5CFU mL^{-1} *E. fergusonii* was co-inoculated in it (post fermentation contamination) then stored for 120 h at 25°C (Figure 3A). After 48, 72, and 96 h of storage, the viable cell counts of the bacterium declined to undetectable level in milk inoculated with concentrations of 0.0625% CL / 0.1875% CE, 0.1875% CL / 0.0625% CE and 0.125% CL/ 0.125% CE respectively. With continued storage, a reduction in the bacterial numbers in the rest of the samples was observed and after 96 and 120 h there was a reduction of approximately 2.5 - 2.3 log units in samples with 0.125% Cl and 0.125% CE respectively. Th pH of the sample continued to decline although slowly with the pH in the range of 4.8 - 5.2 (±0.2) and declined to 4.4 -5.0 (±0.2). Another set of milk was fermented at 37°C for 24 h and then various concentrations of CL mixed with CE were added concurrently with *E. fergusonii* and stored for 5 days at 25°C (Figure 3B). The pH of the sample declined over the period of storage to a pH range of 3.5-2.8. At this temperature, the organism was undetectable in the control samples after 120 h as a consequence of this pH, but a more rapid decline was observed in all treated samples except CE alone.

3.4.2. Survival of *S. typhimurium* during storage in milk fermented at 25°C or 37°C then stored at 25°C: For these samples, milk was fermented at 25°C or 37°C for 24h and then different concentrations of CL and CE singly or in combination were added. These samples were also spiked with approx. 10^5 CFU/mL *S. typhimurium* and then stored at 25°C for 120 h (Figure 4A and B). In milk fermented at 25°C and then stored, *S. typhimurium* declined from the onset to undetectable levels after just after 48 in milk containing 0.0625% CL /0.1875% CE and within 72 h of storage in samples with 0.1875% CL / 0.0625% CE. In milk fermented at 37°C, the decline of the pathogen was even more marked with undetectable levels within 48 h in all treated samples.

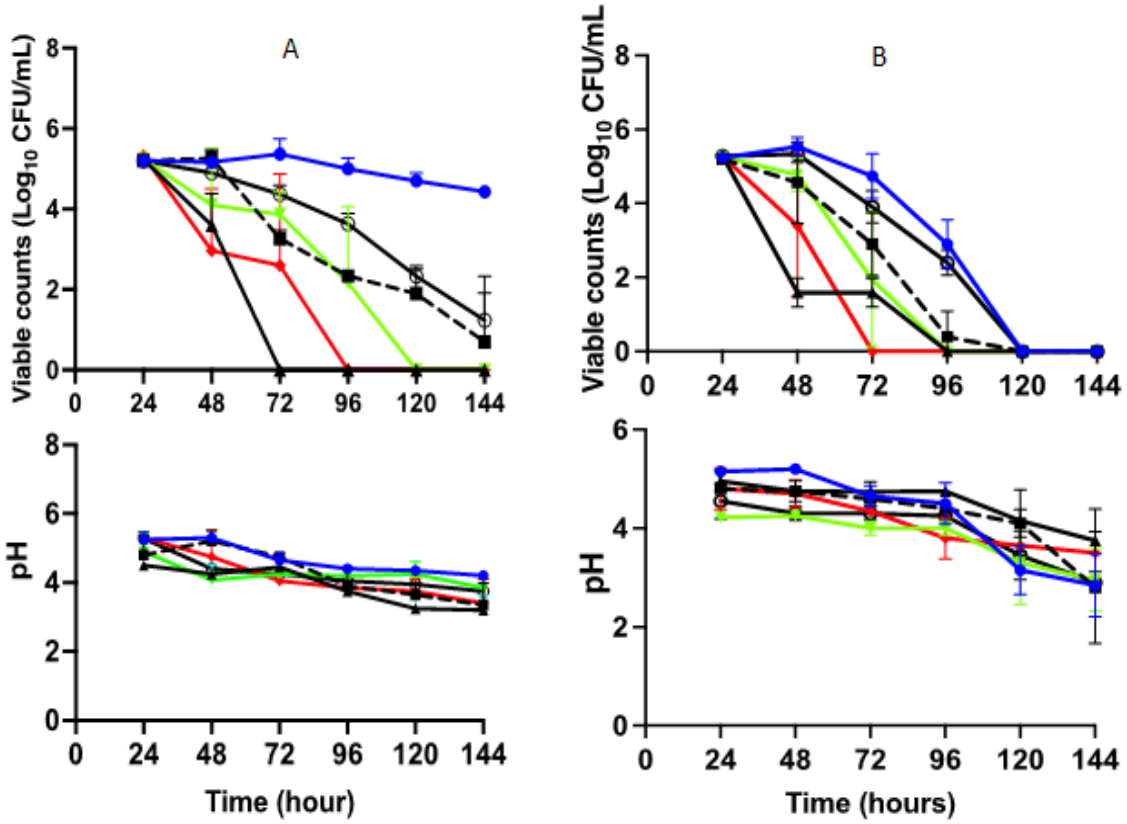

Figure 3: Survival of *E. fergusonii* and change in pH in milk fermented at A) 25°C and B) 37°C and subsequently stored at 25°C for 24 h in the presence of clove (CL) and cinnamon (CE) extracts at different concentrations at. Concentrations are in % (w/v), values are the mean of three individual replicates (means ± SD).

Control [●]; 0.25% CL alone [--■]; 0.0625% CL / 0.1875% CE [-▲-]; 0.125% CL / 0.125% CE [▼]; 0.1875% CL / 0.0625% CE [◆]; 0.25% CE alone [-o-].

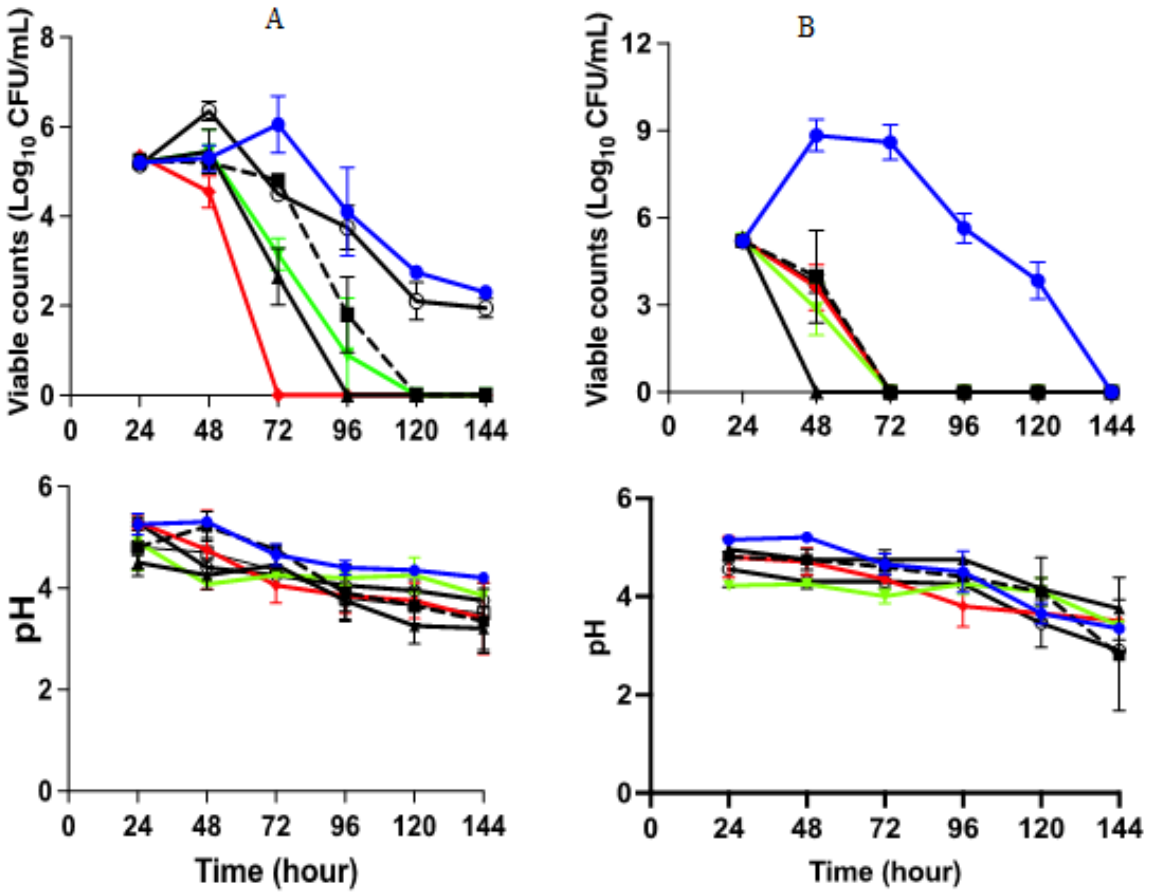

Figure 4: Survival of *S. typhimurium* and change in pH in milk fermented at A) 25°C and B) 37°C and subsequently stored at 25°C for 24 h in the presence of clove (CL) and cinnamon (CE) extracts at different concentrations. Concentrations are in % (w/v), values are the mean of three individual replicates (means ± SD).
Control [●]; 0.25% CL alone [--■]; 0.0625% CL / 0.1875% CE [-▲-]; 0.125% CL / 0.125% CE [▼]; 0.1875% CL / 0.0625% CE [◆]; 0.25% CE alone [-o-].

4. Discussion

4.1. Chemical composition of methanol clove buds and cinnamon bark extracts

Spices have been used all over the world in cuisine and beverages to impart extra flavour. EOs and volatile products of plant secondary metabolism have been used in food industry and medical research. The major constituents in cloves extract were identified as being eugenol (68%), caryophyllene (12.3%); and eugenol acetate (8%) (Table 2) and is agreement with the literature [36-40]. These reports focus on the constituents in cloves from the different places around the world but this the first report on analysis of clove buds sold in the markets in South Sudan. The antimicrobial activity displayed by CL and CE can be assigned to their high content in eugenol or cinnamaldehyde as reported by some researchers [41,42] but also could be influenced by the minor components. Ceylan and Fung [43] reported that phenolic, aldehyde and ketones were the principal compounds of cinnamon EO. However, in 2009, Goni et al. [44] reported that, the major components of cinnamon EO are trans-cinnamaldehyde or cinnamaldehyde. These are consistent with the results

in this study, cinnamaldehyde was the major component with 83.46%. Li et al. [45] and Marongiu et al., [48] identified a range from 66.26-81.97% and 66.28-77.21% in cassia bark respectively. Shan *et al.,* and Chairunnisa et al. [49, 50] reported a lower value of *trans*-cinnamaldehyde (56.10%) in cassia bark whilst Zhang [46] reported the highest content of 92.40%. According to Alma et al. [38] eugenol was 89.6% of clove bud and similar values were reported by Santin et al. [37] with 89.6% of the clove oil was eugenol. The report of Lv et al. [47] reported a slightly lower level of eugenol of 77.45%. In this study eugenol accounted for 68.77% which is consistent with the range reported by Uddin *et al.*, [51] of 60-90% eugenol in cloves from different areas. Hazzit et al., [52]) however, concluded that spices do differ in components even if they were from the same species. Purseglove *et al.,* [53]) reported that post-harvest processing, pre-treatment before extractions as well as the methods of extractions and treatment after the extract all affect the constituents of spices. According to Belcher [54] eugenol content of the oil is dependent on the time taken to distil the product and could vary within 0.41% - 3.11%. Not only the extraction method and *Cinnamomum* species affect the yield, but research have found that also the age and segment (top, center and lower) part of the tree do influence the yield. Besides, there are variations in the methods of extractions of spices or even processing method by different researchers which could have resulted into the variation of the quality and quantity and the constituent compositions of the spice extracts [55, 56].

4.2 Antimicrobial activity of CL and CE by agar well diffusion

The methanol extracts of clove and cinnamon were effective against *E. fergusonii* and *S. typhimurium* with zones of inhibition ranging from 14 to 18 mm. The ZOIs of cloves against *E. fergusonii* or *S. typhimurium* were not significantly different at all the concentrations. There are reports that *E. fergusonii* is an emerging drug resistant pathogen [12,57] but a similar sensitivity to clove extract was found here to *S. typhimurium.* Ampicillin (10 µg) was included for comparison and the ZOI were 14.2±0.5 and 16 ± 0.3 mm for *E. fergusonii* and *S. typhimurium* respectively. According to CLSI [58], resistance is shown when the ZOI to ampicillin (10 µg) are $\leq$ 13 mm; intermediate if ZOI are 14-16 mm and susceptible if $\geq$17 mm. This means both organisms show intermediate susceptibility to ampicillin. In comparison with clove and cinnamon extracts using this scale, at the highest concentration, *E. fergusonii* was susceptible to clove and showed intermediate susceptibility to cinnamon extract. Whereas *S. typhimurium* was susceptible to clove extract and showed intermediate sensitivity to cinnamon extract. In contrast, Galivance [59] reported that with agar well diffusion, cinnamon oil showed a stronger antimicrobial than clove oil against *E. coli* which is a close relative to *E. fergusonii.*

4.3 Effect of combined CL and CE on growth of *E. fergusonii* and *S. typhimurium* and change in pH of milk fermented at 25 or 37°C

The effect of CL and CE on growth and survival of *E. fergusonii* and *S. typhimurium* were investigated during fermentation by the LAB (pre-fermentation contamination). At 25^{o}C, *E. fergusonii* and *S. typhimurium* grew in all the samples during the 24 h of fermentation, although growth was slightly reduced in the samples treated with the spice extracts. The results show that contamination of the milk before fermentation allows the pathogens to grow early in the fermentation when the acidity is low. Acidification is an important measure to control the

growth and survival of pathogens and spoilage microorganisms. However, yoghurt and other acidic foods have been implicated in the foodborne outbreaks caused by pathogens such as *E. coli* O157:H7 [60]. Acid adaptation and increased resistance of pathogens such as *Salmonella* spp. have been reported [61,62]. Similar growth and survival of *Salmonella typhimurium* during fermentation of kefir a traditional fermented milk of Ethiopia was found [63]. *E. fergusonii* is reported as a drug resistant pathogen however, there no report on its resistance to fermentation acids. The temperature of the milk fermentation exerted a marked effect on the bacteria with 37°C resulting to faster reduction in viable numbers of the pathogens than 25°C which was associated with a lower pH due to the acid production at the higher temperature (Figures 1A and B). Reduction of pH during fermentation with the inclusion of the spice extracts also showed that the activities of lactic acid bacteria was not affected. At 37^{o}C, the pathogens were completely inactivated after twenty-four hours of incubation with 0.1875% CL/0.0625% CE and 0.0625% CL/ 0.1875% CE. Inhibition of both *E. fergusonii* and *S. typhimurium* was enhanced due to the more rapid acidification of the milk at the higher temperature by the lactic acid bacteria. The observation in the present study suggests that the use of a higher temperature than the traditional fermentation temperature can restrict pathogen growth and would improve and enhance product quality of the fermented milk. The results suggest that both CL and CE are effective EOs capable of improving the preservation effects of milk fermentation against *E. fergusonii or S. typhimurium*. Clove extract showed a stronger antimicrobial effect than cinnamon extract when applied individually. The fractional combination of clove extract to cinnamon extract showed an additive effect against both *E. fergusonii* and *S. typhimurium*. The extent of the effect depended on the concentration of the EOs. Ogwaro et al. [64] reported similar results when black pepper extract was combined with clove extract. This work has shown that cloves combined with cinnamon enhances the inhibition of the bacteria. According to Moon et al. [65], at low pH, the antimicrobial molecules bind better to the hydrophobic zone of the membrane, where they are diluted in the lipid phase, improving their activities on bacteria and fungi. The increased inhibition at 37^{o}C, the higher temperature, could be due to increased fluidity of the cytoplasmic membrane that occurs at warmer temperatures [65]. This effect is temperature dependent and explain the enhanced effect at the higher temperature. In addition, to temperature of fermentation, the effect of a combination of the spices showed a higher antimicrobial activity compared to a single application of both EOs. These findings may be useful for food applications, but their effect on sensory quality of various foods need to be studied. Moreover, where the amount of CL was higher than CE, the inhibition was increased and indicates that CL may act as a facilitator for enhancing the activity of CE. In an earlier report, Sinenky and Horváth et al. [66, 67] reported the ability of cinnamaldehyde to inhibit amino acid decarboxylases in *Enterobacter aerogenes* and suggested that this could be due to carbonyl group binding to proteins, thus preventing the action of amino acid decarboxylases. Trans-cinnamaldehyde is capable of inhibiting *E. coli* and *Salmonella* by gaining access to the periplasm and inhibiting the activity of transmembrane ATPase as well as without gaining access to the periplasm as well as the deeper parts of the bacterial cell [68]. The effect of the EOs depended on the composition of the mixture between the two oil extracts as well as the temperature of incubation.

4.4 Effect of combined concentrations of CL and CE on the survival of *E. fergusonii* and *S. typhimurium* and change in pH post fermentation

When the antimicrobial was applied post-fermentation then stored, the EOs were more effective than when applied pre-fermentation. This suggest that for optimum pathogen inactivation, the EOs need to be added once the pH has been reduced by the LAB fermentation. It was also observed that applying the EOs post milk fermentation promoted faster inhibitory effects (Figures 3 and 4). The pH decreased further during storage indicating that the lactic acid bacteria continued to be active degrading any lactose which was still remained in the yoghurt. The gradual decrease in pH during storage supports the report of Singh et al., [69] who also reported that acidity of fermented milk increased gradually during storage period of fermented milk. The growth limiting pH could be dependent on several factors, most importantly the acid molecule. Besides, the effect of fermentation temperature, relative oxygen supply as yoghurt becomes anaerobic after the curds are formed and contributes to inhibition of growth. As previously, during storage it was also evident that the antimicrobial activities of clove extract were stronger than that of cinnamon extract. The results also suggest that incorporation of EO into yoghurt (post fermentation) is more effective in inhibiting survival of *E. fergusonii* and *S. typhimurium*, whereas application pre-fermentation of milk resulted to lower inhibitory effect. One main hurdle in using essential oils in fermented milk is that the quantity applied by the farmers are not sufficient as single components besides the negative organoleptic and colour change effects when added in amount sufficient to cause antimicrobial effect. Exploiting synergies between several compounds is suggested as a solution to this problem. Furthermore, during storage over a long period of time, essential bioactive components are lost quickly due to volatilzation, chemical degradation, and certain other physical chemical reactions. The stability, bioavailability, and bioactivity of the bioactive components could be resolved through encapsulation. Encapsulation is already used to preserve unstable food bioactive components, flavoring substances, and essential oil components by preventing their contact with moisture, light, and oxygen. In conclusion, this study has demonstrated that CL in combination with CE inhibited the growth of the pathogens *E.fergusonii* and *S. typhimurium* during fermentation and were more effective at 37 than 25^{o}C. Addition of CL in combination with CE post fermentation where the pH is lower significantly reduced the survival of these pathogens. The use of these extracts both during fermentation and storage have the potential to enhance the microbiological safety of these products.

Author Contributions

B.O. performing methodology and writing original draft. H.G. and B.O. were the main persons responsible for planning the experiments and interpretation of the data; H.G., B.O., L.O.G, and D.H. were involved in the editing, reviewing and preparation of the manuscript. All authors have read and agreed to the published version of the manuscript.

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Data availability statement

The data presented in this study are openly available.

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Conflicts of interest

The authors declare no conflicts of Interest.

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