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**PHYSIOCHEMICAL, PROXIMATE COMPOSITION AND MICROBIAL QUALITY
OF A NOVEL PROBIOTIC YOGHURT FORTIFIED WITH PURPLE-LEAF TEA
(*Camelias sinensis*)**

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ABSTRACT

With the increasing demand of health promoting food products, formulations of yoghurts are changing more towards live probiotic starter culture milk fermentation and fortification with plant-based food ingredients to improve the flavour and nutrition composition. Synbiotic yoghurts fermented with probiotic microbes and fortified with prebiotic food ingredients have become very popular as functional foods due to their scientifically proven bioactive compounds that promote human gut health and alleviate dysbiosis. The aim of the current study therefore, was to assess the physiochemical, nutritional proximate composition and viability of probiotics in formulated yoghurt fortified with a novel purple-leaf tea (*Camelias sinensis*) puree that is rich in anthocyanins, bioactive polyphenols. Whole milk was fermented with probiotic starter culture (*Lactobacillus acidophilus* (LA-5), *Bifidobacterium animalis* subsp. *lactis* -ABT 5) from CH Hansens Limited. Subsequently, the yoghurt was fortified with a novel purple tea puree at the Food and Tea Science Workshop of Karatina University, Kenya. Proximate composition and physiochemical properties (pH and mineral salts profile) were analyzed both in the formulated probiotic yoghurt (PYFPT) and control yoghurt without the tea puree. The viability of the probiotic colony forming microbes was determined using the De Man, Rogosa and Sharpe (MRS) anaerobic bacteria selective culturing agar media. The pH results of PYFPT (4.24 ± 0.04) was not significantly different ($p < 0.05$) to that of the control yoghurt even after 28 days of refrigerated storage. However, proximate composition recorded significant differences except for the crude fiber content (0.01 ± 0.01 %). A higher percent moisture content and crude lipid was recorded in the control yoghurt (88.18 ± 0.01 and (2.11 ± 0.02)) than the PYFPT (78.14 ± 0.04 and 1.33 ± 0.09). Inversely, higher percentage of crude protein, carbohydrates and crude mineral salts were recorded in the formulated PYFPT (7.96 ± 0.27 , 11.17 ± 0.25 ; $0.81 \pm$) than control yoghurt (4.23 ± 0.01 , 5.44 ± 0.04 , 0.59 ± 0.01). A profile of ash content of the added purple tea puree recorded 16 different mineral salts. The macro minerals were the major, with potassium recording the highest content (282 ppm), followed by sodium (121 ppm), magnesium (97.2 ppm) and calcium (96.4 ppm). The microbial anaerobic probiotic count (6.14 - 6.80 log CFU/ml) was not statistically and significantly different between the PYFPT and the control yoghurt after 28 days of refrigerated storage. In conclusion, the study demonstrated that it is feasible to formulate nutritious probiotic yoghurt fortified using purple-leaf tea puree with potential for commercialization.

Key words: Probiotic yoghurt, purple-leaf tea, physiochemical property, proximate composition, probiotics viability



INTRODUCTION

There has been a rising demand for probiotic yoghurts over the last few years largely due to food consumers' awareness of the relationship between diet and the gastrointestinal tract health [1, 2]. Probiotic bacteria, live microorganisms that confer health benefits when consumed in adequate amounts to the host, offer a wide range of health benefits based on the dosage and the individual or mixed strain of starter culture microbes [3]. The health benefits of the probiotics vary in mechanisms from inhibition of gut pathogenic microbes' growth [4, 5, 6, 7] to enhanced phagocytic capacity of immune cells and antimicrobial effect [8, 9]. Some of the major probiotic strains with Generally Recognized as Safe (GRAS) status include *Lactobacillus acidophilus*, *Lactobacillus casei*, *Bifidobacteria* species such as *B. animalis*, *B. bifidum* and *B. longum* [10, 11, 12, 13]. Milk fermented products such as yoghurts are also a rich source of high-quality protein, mineral salts, calcium, potassium, zinc, magnesium, niacin, folate and vitamins B₆, B₁₂ and B₁ [14, 15, 16]. The recommended amount of probiotics for optimal therapeutic benefits is a minimum of 10⁶ colony forming units [17]. The viability of the probiotic microorganism during storage is a major determinant of the potency in food products and its shelf life. Probiotic yoghurt is considered quality and safe for human consumption if stored in refrigerated conditions for the viable microorganisms to remain sufficient to provide the health benefits [18]. Enrichment of probiotic yoghurt with plant-based ingredients is very common not only for improved nutritional composition and flavor, but also as sources of dietary fiber and prebiotics that support probiotic microbes [19, 20, 21].

In general, all processed teas (*Camellia sinensis*) orthodox green, purple, yellow, white or the famous Cut/Crush, Tear and Curl (CTC) black teas are loaded with healthful bioactive compounds in the form of polyphenols (catechins and epicatechins), theaflavins, flavonol glycosides, amino acid-L-theanine, caffeine and theobromine [22, 23]. However, the Kenyan purple leaf tea (TRFK, 306/1), a variety that was cloned at Kenya Agriculture and Livestock Research Organisation (KALRO) Tea Research Institute, Kenya, has all the natural polyphenol compounds found in green-leaf tea, in addition to lots of different purple anthocyanidins (malvidin, peralgonodin and cyanidin 3-O-galactoside) and 1,2-di-O-galloyl-4,6-O-(S)-hexahydroxydiphenoyl-β-D-glucose (GHG) and lower caffeine content compared to black CTC tea [24, 25]. The anthocyanins account for the stronger antioxidant activities with anti-inflammatory, anti-microbial, anti-obesity and neuroprotection [26, 27, 28]. A recent study shows that the purple-leaf tea has potential of prebiotic activity that promote the proliferate growth of beneficial gut microbes while reducing the pathogens [29].

The aim of the present study therefore was to evaluate the quality of a novel probiotic yoghurt fortified with purple-leaf tea (PYFPT) puree in terms of its proximate



composition, physicochemical properties and viability of probiotics after refrigerated storage for 28 days.

MATERIALS AND METHODS

Materials

The milk and purple leaf tea were sourced from Karatina University Farm, while the probiotic yoghurt starter culture from Christine Hansen Limited, Denmark. All the other chemicals for analysis were sourced from Sigma-Aldrich Limited supplier in Kenya.

Preparation of the probiotic yoghurt fortified with purple tea (PYFPT)

The novel probiotic yoghurt, fortified with purple tea puree was produced by fermentation of the whole milk with probiotic yoghurt starter culture at the Food and Tea workshop of Karatina University, Kenya. The probiotic starter culture (ABT 5) is composed of *Lactobacillus acidophilus* (LA-5), *Bifidobacterium animalis* subsp. *lactis* BB-12 (BB-12) and *Streptococcus thermophilus*. Subsequently, the yoghurt was then fortified with the formulated novel purple tea puree. The control probiotic yoghurt was prepared without fortification with purple leaf tea puree.

Proximate composition analysis

The proximate composition of the probiotic yoghurt fortified with purple leaf tea (PYFPT) and control samples were analyzed in duplicate according to the standard Association of Official Analytical Chemists (AOAC) analytical methods [30] at the Food Science Laboratories of Jomo Kenyatta University of Agriculture and Technology, Kenya.

Determination of moisture Content

The moisture content of the experimental PYFPT and control probiotic yogurts were determined by drying the samples to constant weight in an oven at 105° C. The loss in weight after drying was determined and recorded as percentage moisture content:

$$\% \text{ moisture} = W_1 - \frac{W_2 \times 100}{W_1}$$

Where W_1 is the initial weight of the sample and W_2 is the weight of the dried sample. Determination of crude protein content.

Total crude protein was analyzed using the Semi-Micro Kjeldhal method as nitrogen content. The crude protein was calculated as:

% Crude protein = % nitrogen × 6.25 (Based on assumption that proteins contained 16% of N).

$$\text{Percentage Crude Protein (\%)} = \frac{0.00014 \times V \times 100 \times 250 \times 6.25 \times 100}{Z \times W}$$



Where

V = Volume of 0.01N H₂SO₄ neutralized by NH₃

W =Weight of the sample (g)

Z = Volume of aliquot taken for distillation

Determination of crude fiber

The crude fiber was determined according to the AOAC procedure [31] as the fraction remaining after the chemical digestion with sulfuric acid (H₂SO₄) and sodium hydroxide (NaOH).

Determination of crude mineral salts (Ash content)

The amount of ash of the yoghurt samples was analyzed by direct incinerated heating in a furnace for 3 hours at 550°C into ash. The ash percentage was calculated using the following formula.

$$\% \text{ ash} = \left(\frac{Y - X}{Z - X} \right) \times 100$$

Where Y = weight of the crucible + ash; X = weight of the empty crucible; Z =weight of the crucible + sample

Crude lipid (Fat) content determination

The crude lipid content was determined using Bligh and Dyer method as described by Soxhlet method. The percentage crude fat was calculated using the formula below:

$$\% \text{ fat extracted} = \frac{\text{weight of extracted fat (g)}}{\text{weight of sample used (g)}} \times 100$$

Mineral elements profiling by acid digestion technique and spectrophotometry

The purple tea puree crude mineral salts (ash) was used to determine the individual mineral profile content by a two- step acid digestion method to remove the organic matter using 5 Molar Nitric acid and concentrated Hydrochloric acid followed by ashing in muffle furnace for 2 hours at 480° C. The solution was filtered through an ashless filter paper and then quantitatively transferred to a 25 mL volumetric flask through adding deionized distilled water. Atomic absorption spectrophotometer was used to determine the mineral content profile against the individual standard minerals.

Determination of carbohydrate content

The percent of carbohydrates (% CHO) was obtained by deducting the summation of % (crude fiber, protein, ash, moisture and lipid) from 100

$$(\text{Carbohydrate}) = 100 - (\text{Protein} + \text{Fat} + \text{Crude fiber} + \text{Ash})$$



Microbial quality of the purple tea probiotic yoghurt

The microbial quality of the yoghurts (PYFPT and control) were determined by viable enumeration count of aerobic mesophilic bacteria using the International Organization for Standardization method (ISO 15214:1998) [31] while the anaerobic probiotic microbes (Lactic acid bacteria and Bifidobacteria) by selectively culturing in De Man Rogosa and Sharpe (MRS) agar media. The inoculated plates were incubated at 30-35° C and 42° C, respectively for 48 hours. The characteristic bacteria colonies on the plates between 30 and 300 were counted and multiplied by their respective dilutions to represent colony forming units per milliliter (CFU/ml).

Determination of pH

A potentiometric method was used to determine pH in triplicate using a pH meter calibrated using buffer solutions of pH 4 and 7.

Determination of keeping storage quality of the yoghurts

The storage quality of the PYFPT and control yoghurt was evaluated weekly by analyzing pH and microbial probiotic viable count after refrigerated storage at 4°C for 28 days.

Statistical analysis

All collected data was subjected for analysis using Statistical Package for Social Sciences (SPSS) Version 20. Analysis of variance (ANOVA) was tested for any statistically significant difference ($p < 0.05$), while Duncan's multiple range test was used to separate means at significant differences of ($p < 0.05$) for the PYFPT and control yoghurts.

RESULTS AND DISCUSSION

Physiochemical properties of the probiotic yoghurts

The PYFPT had an appealing light purple color while the control yoghurt was white. There were no observable changes that affected the quality of both yoghurt samples such as syneresis (separation of the whey). Syneresis lowers the sensory quality of yoghurt in terms of texture and appearance, thus leading to rejection by the food consumers [32, 33]. The mean pH of the PYFPT and control yoghurts (4.23 ± 0.02) was not significantly different between the PYFPT and control yoghurt (Figure 2).

Proximate composition

The probiotic yoghurt fortified with purple tea leaf (PYFPT) and control probiotic yoghurt without purple leaf tea had significant difference in composition except for the crude fiber content at (0.01 ± 0.01 %) (Table1). The amount of fiber may have remained the same because the puree was sieved before adding to the yoghurt. There is need to consider incorporating some of the tea fiber during formulation of the puree to improve the yoghurt nutritional value. The percent moisture content of



PYFPT (78.14%) and crude lipid (1.33%) were the only variables that were lower compared to the control yoghurt (88.18% and 2.11%), respectively. On the contrary, the percent crude protein, carbohydrate and ash content of PYFPT were higher at (7.969%, 11.7% and 0.81%) while that of the control yoghurt were at (4.24%, 5.44% and 0.59%), respectively. These differences may be attributed to the chemical composition of purple tea leaves [25]. Various other related studies reported changes in yogurt nutritional value after fortification with other types of tea especially in the content of ash and protein. For example, yoghurt fermented with different starter culture microbes and fortified with green tea recorded changes in the moisture, ash, fat and protein contents [34]. On the contrary, drinking yoghurt incorporated with green tea extract recorded significant differences in protein content [35]. A comparative study between green tea (*Camellia sinensis*) and damgatondam upland rice grass leaves demonstrated that processing technology (steaming or panning) and conditions (temperature/time) resulted in variation in proximate composition and micronutrients (Calcium, Magnesium, Potassium, Iron, Manganese and Sodium) [36].

Profiling of Mineral Salts Content in Purple Tea Puree

The study found that the mineral content analyzed from purple tea puree (Figure 1) had the highest macro mineral, potassium at (282 mg/L) and worth noting about twice in concentration than that of sodium. The amount of magnesium and calcium followed. The trace minerals manganese, aluminum and iron had the lowest concentrations. The study further noted that the trace minerals zinc, copper, boron, and chromium contents were below 10 mg/L. These variations in mineral salt concentrations were also reported in other previously related tea studies. A study on ready-to-drink ice tea (black, green, mate and white tea) found variation in trace mineral salts profile whose bio-accessibility and bioavailability was influenced by the level of tannins in the tea beverages. Besides the health risk implications of tannins, the tea country of origin, type, and infusion process also has a significant influence on the trace element contents in tea leaves [37].



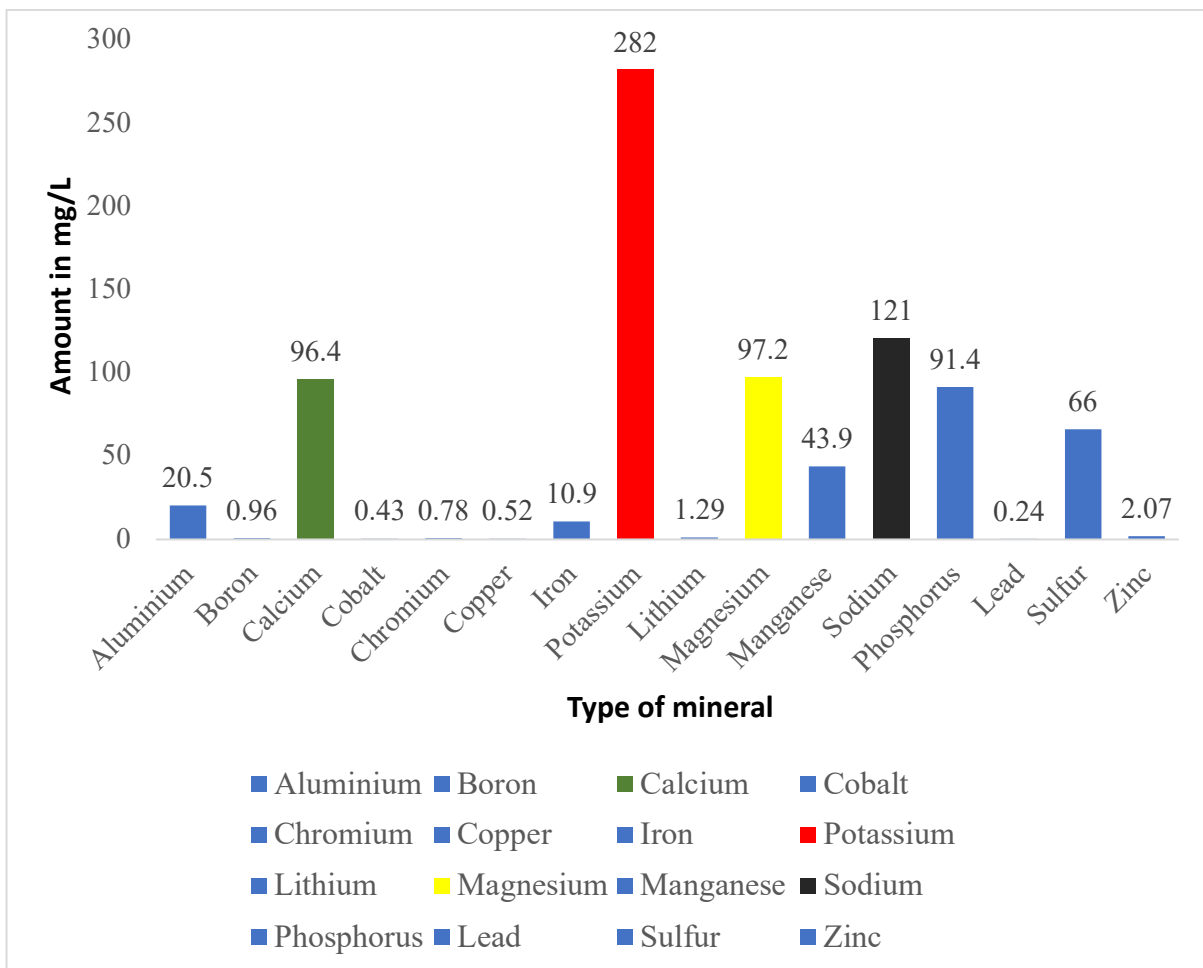


Figure 1: Mineral content profile of purple tea puree in ppm

Properties of the yoghurts after refrigerated storage

Change in pH of the yoghurts

The mean pH of the PYFPT (4.23 ± 0.02) was not significantly different ($p= 0.24$) from the control yoghurt even after 28 days of refrigerated storage (Figure 2). This finding contradicts a previous related study whereby variations were reported due to post-acidification during refrigerated storage [38]. Acidity/pH changes have been reported to negatively influence the sensory and shelf life quality yoghurt in terms of taste and syneresis (water-holding capacity) and viscosity [39, 40].

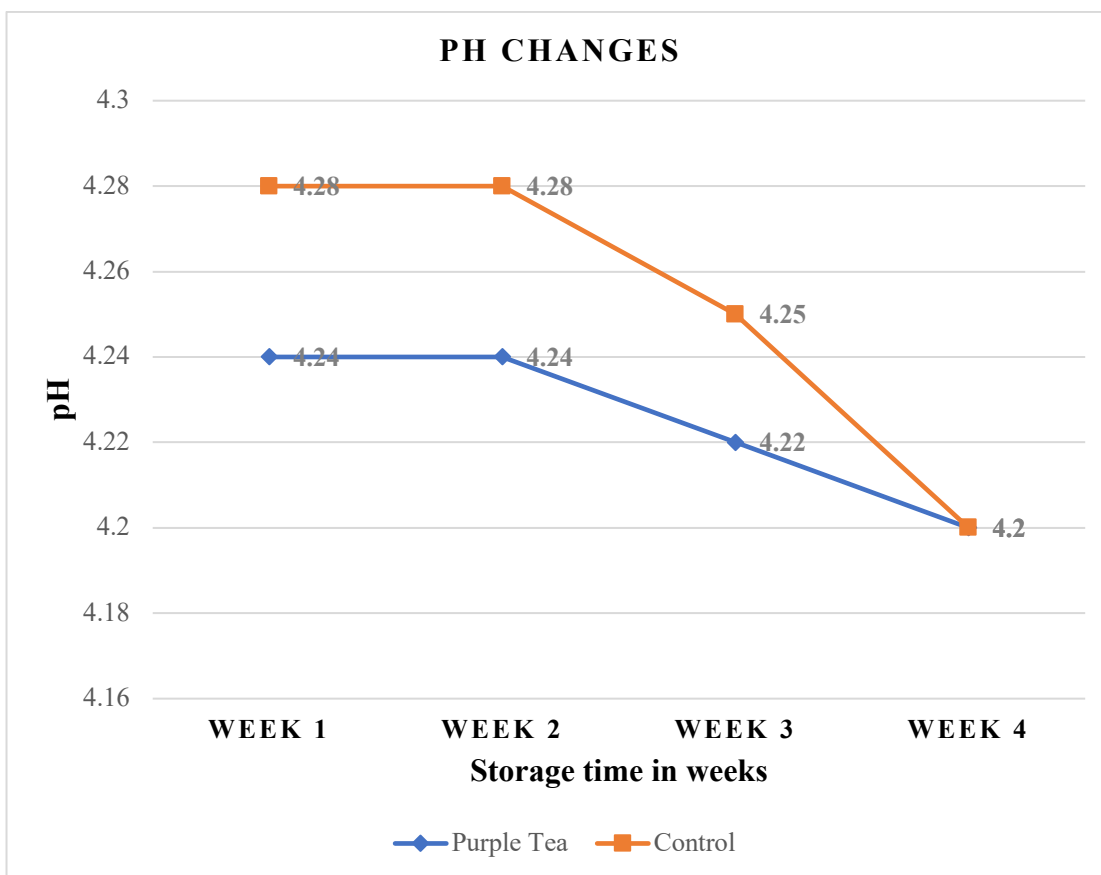


Figure 2: pH of PYFPT and control yoghurt over 4 weeks of refrigerated storage

Microbial quality of the yoghurt after refrigerated storage

After refrigeration storage at about 4° C for 28 days, both the purple tea yogurt (PYFPT) and control yoghurt had no significant differences in the aerobic mesophilic bacteria count (Figure 3). The total viable count, coliform, yeast and mold count (10^{1-2} , zero and 2-3 CFU/ml, respectively) were within the recommended levels by Kenya Bureau of Standard-ISO 4833-2:2013(E). This is a good pointer that hygienic conditions were maintained during the yoghurts' production.

TPC, Coliform and Mold / Yeast plates



Figure 3: Total plate count (TPC), coliform, yeast and mold plates, respectively after incubation

Viability of the probiotic bacteria culture microbes in the purple-leaf yoghurt

The anaerobic probiotic bacteria colony forming units per milliliters (log of CFU/ml) at start of experiment of the PYFPT was at 6.46 Log CFU/ml, which increased after the first week to 6.69 Log CFU/ml (Figure 4). Subsequently, there was a decrease to 6.24 and 6.14 Log CFU/ml in the second and third week, respectively and an increase in the fourth week to 6.80 Log CFU/ml. However, in the current study, these changes were not significantly different ($p= 0.97$) from the first week to the end of the 4 weeks of the refrigerated storage period. No significant growth was observed on the control yoghurt plates (Figure 4 and 5). The results suggest that purple tea may have prebiotic ingredients that support the survival of the probiotic bacteria in the yoghurt. Furthermore, the viable count remained within the recommended number of at least 10^6 CFU/mL for human therapeutic benefits [17]. In a related study, Pu-erh tea polyphenols have been reported as having prebiotic activities that support proliferation and survival of healthy gut microbes [41].

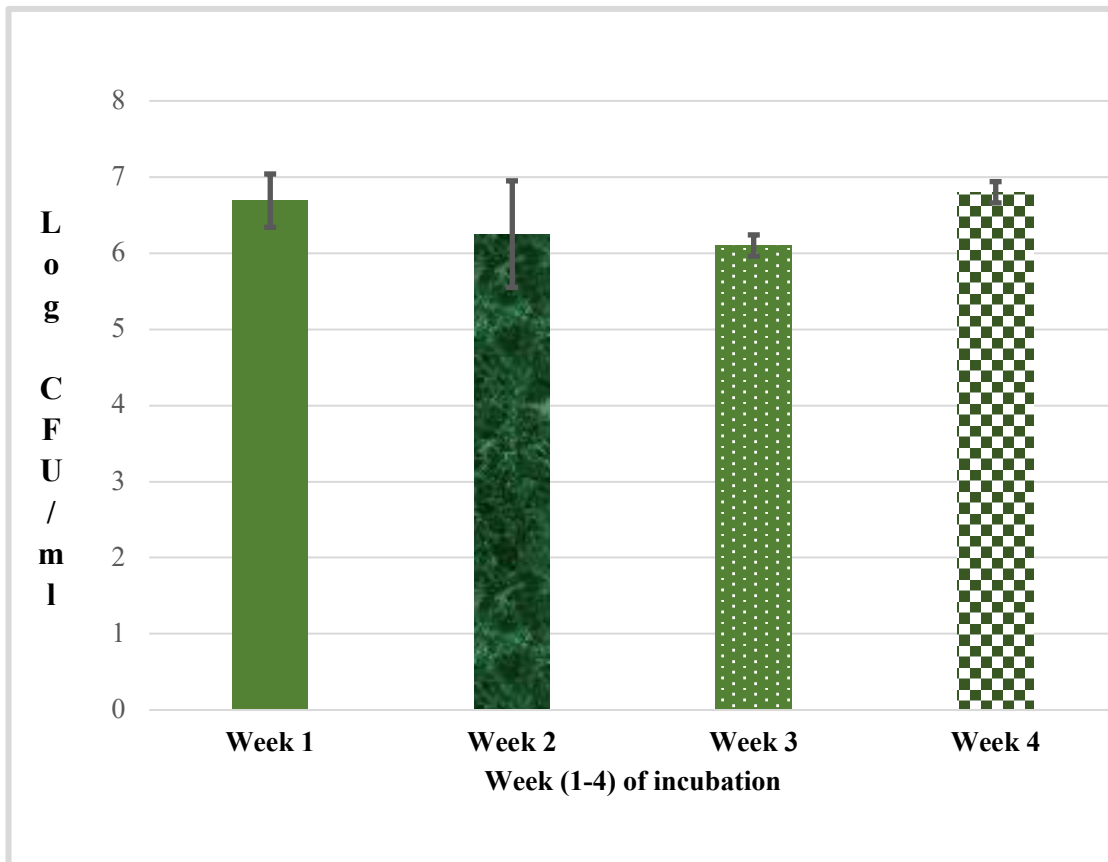


Figure 4: Anaerobic probiotic bacteria count (CFU/ml) of PYFPT in the four weeks of refrigerated storage

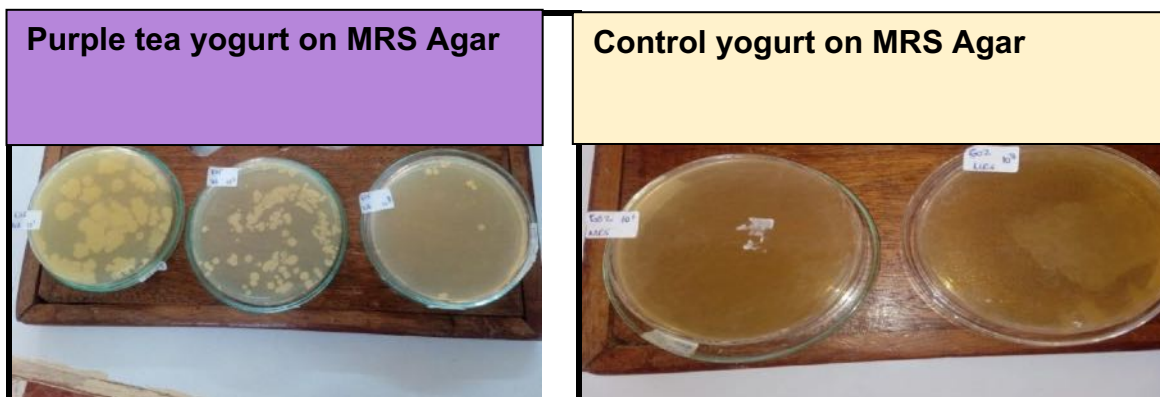


Figure 5: Anaerobic probiotic bacteria growth in purple tea yoghurt and control yoghurt on MRS agar plates

The greatest concern in development of novel yoghurts is the survival of probiotic microbes during processing and storage with no detectable pathogenic microbes [42, 43].

CONCLUSION AND RECOMMENDATIONS FOR DEVELOPMENT

The research demonstrates that the formulated probiotic yoghurt fortified with novel purple leaf tea puree has extra nutritional benefits in terms of protein and mineral salts. The pH of the purple tea yoghurt and control yoghurts did not significantly differ, even after 28 days of refrigerated storage. Moreover, there was no observable syneresis of the yoghurt. The research outcome also shows that the probiotic starter culture microorganisms survived in the formulated yoghurt within the recommended standards despite the antimicrobial properties of the purple leaf tea. However, there is need to conduct further investigation on prebiotic activity of the yoghurt that may influence viability of beneficial bacteria in the human gut. In addition, before launching the yoghurt commercially in the market, studies are required to establish its shelf life and consumer acceptability of the sensory attributes.

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Conflict of Interest

The author declares that they have no conflict of interests.

Authors' contributions

All authors contributed to the design, drafting, reading and approval of the final manuscript.



Table 1: Proximate composition of PYFPT and Control yoghurts

Parameters	PYFPT	Control
Moisture (%)	78.14±0.04	88.18±0.07
Crude Protein (%)	7.97±0.27	4.24±0.01
Carbohydrates (%)	11.17±0.25	5.44±0.04
Crude fiber (%)	0.01±0.01	0.01±0.01
Crude mineral (Ash) (%)	0.81±0.01	0.59±0.01
Crude lipid (%)	1.33±0.09	2.11±0.02

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