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VIABILITY AND EFFECT OF 'PROBIOTIC' LACTIC ACID BACTERIA ISOLATED FROM LOCALLY FERMENTED FOODS IN A NON-DAIRY BEVERAGE FROM AFRICAN YAM BEAN

H. A. Adeniran
Department of Food Science and Technology
Obafemi Awolowo University, Ile-Ife, Nigeria

O. I. Olaniyi
Department of Food Science and Technology
Obafemi Awolowo University, Ile-Ife, Nigeria
busayoolaniyi1702@gmail.com

S. H. Abiose
Department of Food Science and Technology
Obafemi Awolowo University, Ile-Ife, Nigeria

ABSTRACT

This study assessed the feasibility of using African yam bean (AYB), a legume not well known in Nigeria, as a non-dairy probiotic beverage. Lactic acid fermentation of milk blends from AYB, coconut and soybean milk was carried out and samples were stored at ambient (28±2 °C) and refrigerated (4±2 °C) temperatures. The samples were observed for 28 days at 7 days interval. Viability of the lactic acid bacteria (LAB) was done using standard methods and changes in pH, titratable acidity (TTA), total amino acid and total reducing sugar were monitored. The LABs were found to be viable at both storage temperatures. The pH of samples decreased, TTA increased, total amino acid varied among samples while total reducing sugar decreased over storage period. It was concluded that non-dairy beverage from the three vegetable materials could be stored in the refrigerator for 28 days and 21 days at ambient temperature without unfavourable effect on its inoculated LAB population and physicochemical properties.

Keywords: *Non-dairy, African yam bean, Probiotic, Lactic acid bacteria, Storage, Viability*

1. INTRODUCTION

Over the years, dairy milk has been the only suitable substrate exploited as probiotic carrier. For some reasons such as its affordability and religious purpose, recent development has shifted to using non-dairy substrates as probiotic carriers. Vegetable milks such as peanut milk, tiger-nut milk, cowpea milk, and soymilk have been explored (Adeniran *et al*., 2010; Ebhodaghe *et al*., 2012). Under-exploited plants such as African Yam Bean (AYB) which has high crude protein content (23%) (Fasoyiro *et al*., 2012) has also been used as a source of vegetable milk (Aminigo *et al*., 2007).

Probiotic foods are a group of functional foods with growing market shares and large commercial interest (Arvanitoyannis and Van Houwelingen-Koukaliaroglou, 2005). Probiotics contain live microbes that can be incorporated into many different types of products such as foods, drugs and dietary supplements. Probiotic is a relatively new term (Lilly and Stillwell, 1965) used to describe the bacteria associated with the beneficial effects for the humans and animals (Song *et al*., 2012). The term probiotic means "for life" and it was defined by an Expert Committee as "live microorganisms which upon ingestion in certain numbers exert health benefits beyond inherent general nutrition" (FAO/WHO, 2001).

Lactobacillus and *Bifidobacterium* are the most commonly used probiotics in food and feed. Since ancient times, Lactic acid bacteria (LAB) have been used for food fermentation and can also serve a dual function of acting as food fermenting agent and potentially health benefits provider (Song *et al*., 2012). LAB are known as GRAS (generally recognized as safe) with no pathogenic or virulence properties been reported. Some desirable characteristics of LAB such as low cost, maintaining its viability during the processing and storage, and resistance to the physicochemical processing must be ascertained before it is used as probiotics.

This study investigated viability of LAB with probiotic potential in African yam bean milk with or without soymilk and/or coconut milk stored at ambient and refrigeration temperatures. It also looked into the effect of such LAB on the physicochemical characteristics of the fermented milk with the view to establishing the desirability or otherwise of using the non-dairy vegetable milk as a good alternative to those that will not consume dairy milk based fermented milk for different reasons.

2. MATERIALS AND METHODS

2.1. Materials

Vegetable milk samples were produced from three base materials; AYB, soybean and coconut. These plant produces were purchased from a retailer in a local market in Ile-Ife, Osun State, Nigeria. Probiotic organisms were sourced from locally fermented foods such as *ogi*, *wara* and *nunu*. AYB milk (AYBM), Coconut milk (CM) and soymilk (SM) were produced as described by Aminigo *et al*. (2007), Sanful (2009) and Udeozor *et al*. (2012) respectively. The vegetable milk samples were formulated into proportions 1:1:1 and 2:1:1 (AYBM: CM: SM). The milk blends were then fermented with individual strains and a combination of the organisms in an incubator at 45 °C for 18 h (Amakoromo *et al*., 2012).

The fermented milk samples were refrigerated for an h before bottling in sterile sample bottles. A set of the samples were stored

at ambient temperature (28 ± 2 °C) and refrigerated temperature (4 ± 2 °C) for 28 days. Microbial analyses were carried out at an interval of 7 days for a period of 28 days using methods of Harrigan and McCance (1976) and Harrigan (1998).

Sorghum grains were cleaned and soaked for 48 h at 28 ± 2 °C, rinsed with water, wet-milled and sieved. Supernatant of the fermented *ogi* (1.0ml) was transferred to 9ml sterile 0.1% peptone diluents. The tubes were each shaken thoroughly for 10 seconds to ensure homogenization. Each dilution (1.0ml) was plated out on MRS agar using pour plate technique (Harrigan and McCance, 1976; Harrigan, 1998). The molten agar was allowed to set, the plates inverted and incubated at 37 ± 2 °C for 48 h.

2.2. Isolation of LAB from *Nunu*

After *nunu* had been serially diluted, 1 ml of each dilution was plated on MRS agar using pour plate technique (Harrigan and McCance, 1976; Harrigan, 1998). The medium was allowed to set; plates were inverted and incubated at 37 ± 2 °C for 48 h.

2.3. Identification of LAB

Representative colonies of LAB were picked from incubated plates and, purified further by repeated streaking on solidified MRS agar plates. The pure cultures of the isolates were maintained on MRS agar slants in McCartney bottles and kept at refrigeration temperature (4 ± 2 °C). Pure isolates were identified according to scheme and procedure described in Harrigan, 1998. Gram reaction, catalase test, nitrate reduction and other biochemical tests were performed to identify the isolates.

2.4. Preparation of fermented milk from blends of AYB, Coconut and Soybean milk

Milk samples from the plant sources were proportioned into 1:1:1 and 2:1:1 (AYBM: CM: SM) and then sterilized at 121°C for 10 mins in an autoclave. Culture suspensions of *Lactobacillus paracasei* and *P. pentosaceus* were prepared and absorbance adjusted to 1.0 at 540nm using a spectrophotometer (Omafuvbe *et al*., 2002) under aseptic condition. LAB strains were reactivated in skim milk (12% w/v) and inoculated into the sterilized milk individually and a combination of both strains in ratio 1:2 *P. pentosaceus to Lactobacillus paracasei*. Samples were fermented in an incubator at 45 °C for 18h (Fig. 1). Fermented milk samples obtained were evenly distributed into sterile bottles and stored at ambient and refrigerated temperatures. Viability of these strains was monitored for 28 days at 7 days interval.

2.5. Estimation of LAB in fermented product

Microbial counts of the LAB present in the samples were observed at the initial and on a weekly basis during storage period. Serial dilution of each sample stored at both temperatures was carried out and from each dilution, 1ml was plated using pour plate technique and molten MRS agar was added. The plates were incubated at 37 °C for 48-72h. Plates with colonies between 30-300 colonies were counted using Gallenkamp colony counter (NW-300 Model). The experiment was carried out in triplicate and mean of counts was determined in each case.

2.6. Determination of pH

The pH (hydrogen ion concentration) of the fermented milk samples was determined using a standard pH meter (ATC, Model HI-8915) (AOAC, 2000).

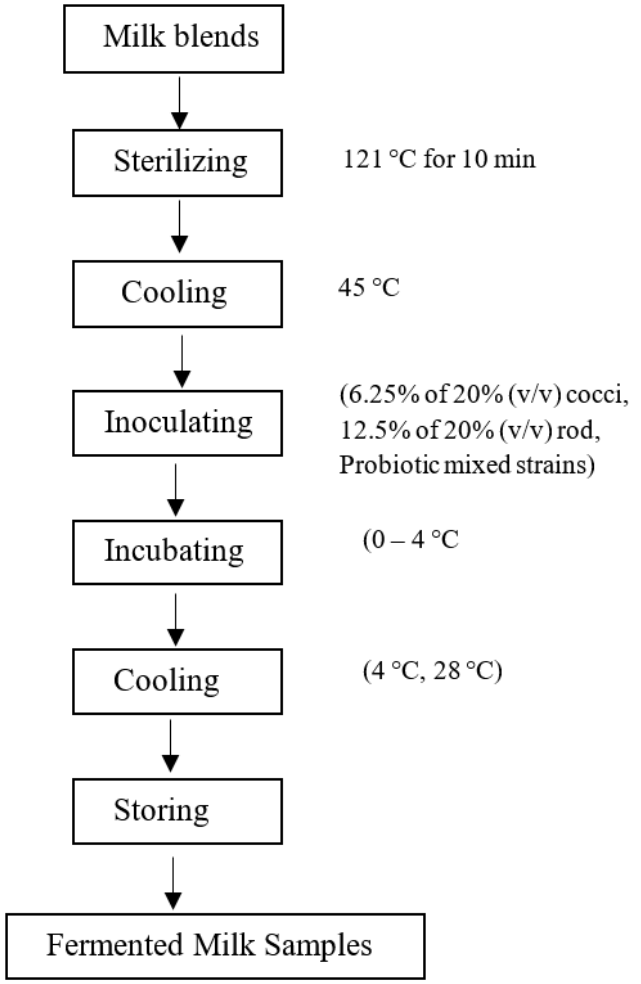

Fig. 1: Fermentation of Selected Milk Blends
Source: Adapted from Amakoromo et al. (2012)

2.7. Determination of Titratable acidity

The production of lactic acid was determined by titrating 10 ml of the homogenized sample diluted with 10ml distilled water against 0.1N NaOH using 0.5 ml of phenolphthalein indicator (0.5% in 50% alcohol). The mixture was shaken thoroughly and titration was carried out until a permanent pink colour was observed in each sample. The titratable acidity was calculated (equation 1) as percentage of lactic acid (v/v). Each millilitre of 0.1 N NaOH is equivalent to 0.009g of lactic acid (AOAC, 2000).

$$\text{Lactic acid (mg/g)} = \frac{ml\ NaOH \times 0.009}{ml\ of\ sample} \quad (1)$$

0.1N x 0.09 = 0.009

2.8. Determination of Total amino acid content

Amino acid content was determined using the formol titration method as described by Frais (1972). Ten milliliter of 40% formaldehyde solution was mixed with 20 ml of distilled water in a beaker, 3 - 4 drops of phenolphthalein indicator was added and shaken thoroughly and neutralized with 0.1M NaOH solution to obtain the faintest permanent pink colour. To 25ml of the fermented milk sample in a beaker, was added 3-4 drops of phenolphthalein indicator was added and neutralized with 0.1M NaOH solution until a faintest permanent pink colour was obtained. The neutralized formaldehyde solution was then added

to the neutralized sample and mixed. The faint pink colour disappears. The sample is then titrated against standard 0.1 M NaOH until a pink colour was produced as before. The titre value was recorded and this was carried out in duplicate. Total amino acid was calculated.

2.9. Determination of reducing sugar content

The method described by Abiose and Adedeji (1994) and Adeniran and Abiose (2011) was used. The fermented milk sample was made up to 4 times its original volume (4-fold dilution) and mixed thoroughly. This was then filtered through a 90mm Whatmann filter paper. One milliliter of the clear filtrate was taken into a test tube and 2ml of DNSA reagent was added. The mixture was boiled for 5 min and cooled under running tap water. Seven milliliters of distilled water was added to make up to 10ml. The absorbance was read against reagent blank at 540 nm in a UV spectrophotometer. Amount of reducing sugars in each sample was extrapolated from the standard glucose curve.

2.10.Statistical analysis

Results obtained as triplicates were subjected to analysis of variance while Duncan's multiple range test was employed to separate the means.

3. RESULTS AND DISCUSSION

3.1. Viability of the LAB strains in non-dairy carrier

The initial LAB count before storage ranged from 6.52 to 9.17 (log cfu/ml) (Tables 1a and 1b). Equal proportion of milk blend fermented with *Lactobacillus paracasei* (Sample AR) had the highest initial count of 9.17 (log cfu/ml) which decreased after storage to 7.58 and to 7.59 (log cfu/ml) at ambient and refrigerated temperatures respectively. After 21 days of storage, the LAB counts of milk blend 2:1:1 fermented with *P. pentosaceus* (sample BC) (4.56 log cfu/ml) and milk blend 2:1:1 fermented with *L. paracasei* (BR) (4.28 log cfu/ml) were below the acceptable probiotic limit (6 log cfu/ml). Depletion in nutrients available for microbial growth could be the possible reason for this observation. Also, Milk blend 1:1:1 fermented with combination of both strains (Sample ARC) at refrigerated temperature showed 3.7% increment in LAB count. Suitable growth condition due to synergism of the isolates could be the reason for the increase observed. Sample AYBF (100% AYBM with commercial starter culture) had the lowest initial LAB count (6.52 log cfu/ml) which reduced to 5.58 log cfu/ml at ambient storage and to 6.51 log (cfu/ml) at refrigerated temperature after 28 days. Ebhodaghe *et al.* (2012) reported a decrease in *Bifidobacteria longum* count in probiotic soymilk after 21 days of storage at ambient and refrigerated storage. Decrease in population of probiotic strains after storage could be due to decreasing pH, increase in acidity of the medium, nutrient depletion such as carbohydrate and protein required for optimal microbial growth (Prescott *et al*., 1999). Viability of *Lactobacillus acidophilus* and *L. plantarum* in roselle extract for 27 days of storage at ambient temperature was reported by Aramide *et al.* (2009). All probiotic strains were viable at both storage temperatures after 21 days.

Lactobacillus paracasei L27 showed a stable level of viable cells in fermented milk sample with blend proportion 2:1:1 (91.5% and 95.2% viability) while combination of *L. paracasei* L27 and *Pediococcus pentosaceus* RS15 in fermented milk with equal proportion of milk as starting premix showed the highest stability (98.3% viability) at refrigerated temperature. Daneshi *et al.* (2013) reported that *L. acidophilus* LA5 showed a stable level of viable cells (98.8% viability) in milk/carrot juice drink during storage.

The rate of proliferation of organisms in samples stored at 28 °C was observed to be higher compared with samples stored at 4 °C during 28 days storage. There was no significant difference ($P<0.05$) in LAB population after 7 days of storage at both ambient and refrigerated temperature. This could imply that little or no microbial growth occurred during this period due to sudden cold shock experienced by the organisms and also inhibition of most microbial enzymes at low temperatures in thermophilic and mesophilic organisms (Prescott *et al*., 1999; Adeniran *et al*., 2010) in the refrigerated samples. At both storage temperatures, an initial lag growth phase was experienced. *P. pentosaceus* exhibited high population count after 21 days of storage while *L. paracasei* showed high counts between days 0 and 7. At the end of storage, no significant difference ($P<0.05$) was observed in the viability of individual strain and their combination in all fermented milk samples at refrigerated storage. This implies that shelf life stability of fermented milk samples was obtained when stored in a refrigerator for 28 days.

Table 1a: Lactic Acid Bacteria Counts in Fermented Milk Samples during Storage for 28 days at Ambient Temperature

Storage days	AC	AR	ARC	BC	BR	BRC	AYBF
0	8.60 ± 0.01^{b}	9.17 ± 0.03^{a}	8.68 ± 0.01^{b}	8.58 ± 0.07^{b}	7.86 ± 0.02^{c}	8.79 ± 0.08^{ab}	6.52 ± 0.04^{d}
7	8.75 ± 0.02^{b}	8.81 ± 0.02^{a}	8.76 ± 0.01^{b}	8.41 ± 0.01^{d}	8.20 ± 0.02^{e}	8.68 ± 0.06^{c}	8.26 ± 0.03^{e}
14	8.66 ± 0.05^{b}	8.53 ± 0.04^{c}	8.65 ± 0.07^{b}	8.58 ± 0.01^{c}	9.15 ± 0.04^{a}	8.38 ± 0.04^{d}	8.41 ± 0.01^{d}
21	8.95 ± 0.07^{a}	7.85 ± 0.01^{d}	8.11 ± 0.04^{b}	7.95 ± 0.01^{c}	7.82 ± 0.04^{d}	8.15 ± 0.07^{b}	7.81 ± 0.01^{d}
28	8.08 ± 0.01^{a}	7.58 ± 0.01^{b}	7.39 ± 0.01^{c}	4.56 ± 0.02^{e}	4.28 ± 0.10^{f}	7.51 ± 0.01^{bc}	5.58 ± 0.06^{d}

Table 1b: Lactic Acid Bacteria Counts in Fermented Milk Samples during Storage for 28 days at Refrigerated Temperature

Storage days	AC	AR	ARC	BC	BR	BRC	AYBF
0	8.60 ± 0.00^{b}	9.17 ± 0.03^{a}	8.68 ± 0.01^{b}	8.58 ± 0.07^{b}	7.86 ± 0.02^{c}	8.79 ± 0.08^{ab}	6.52 ± 0.04^{d}
7	8.62 ± 0.01^{c}	8.23 ± 0.01^{d}	8.61 ± 0.02^{c}	8.69 ± 0.01^{b}	7.90 ± 0.02^{e}	8.95 ± 0.01^{a}	6.41 ± 0.05^{f}
14	8.59 ± 0.02^{c}	8.56 ± 0.02^{c}	8.85 ± 0.04^{a}	8.85 ± 0.01^{a}	7.98 ± 0.06^{d}	8.77 ± 0.02^{ab}	6.68 ± 0.04^{e}
21	7.98 ± 0.01^{de}	8.08 ± 0.01^{cd}	8.71 ± 0.02^{a}	8.16 ± 0.01^{c}	7.93 ± 0.01^{ef}	8.39 ± 0.01^{b}	6.86 ± 0.06^{g}
28	7.84 ± 0.02^{c}	7.59 ± 0.05^{d}	8.69 ± 0.03^{a}	7.48 ± 0.03^{de}	6.48 ± 0.05^{f}	8.11 ± 0.01^{b}	6.51 ± 0.03^{f}

LAB counts expressed as log cfu/mL Means with the same superscript within rows are not significantly different at $P < 0.05$.

Keys : AC: Equal proportion of milk blends fermented with P. pentosaceus; AR: Equal proportion of milk blends fermented with L. paracasei; ARC: Equal proportion of milk blends fermented with combination of L. paracasei and P. pentosaceus; BC: Milk blend proportion 2:1:1 fermented with P. pentosaceus; BR: Milk blend proportion 2:1:1 fermented with L. paracasei; BRC: Milk blend proportion 2:1:1 fermented with combination of L. paracasei and P. pentosaceus; AYBF: 100% AYB milk fermented with commercial starter culture.

Table 2a: Changes in pH of Fermented Milk Samples during Storage at Ambient Temperature

Storage days	AC	AR	ARC	BC	BR	BRC	AYBF
0	4.13 ± 0.02^{c}	5.49± 0.01^{a}	4.10± 0.01^{c}	4.03±0.03 ^{d}	4.34±0.02 ^{b}	3.75± 0.02^{e}	4.28±0.03 ^{b}
7	3.49±0.03 ^{c}	3.61± 0.01^{b}	3.41±0.02 ^{d}	3.33± 0.02^{e}	3.01±0.02 ^{d}	3.04± 0.01^{f}	3.67± 0.02^{a}
14	3.54±0.02 ^{c}	3.83± 0.01^{b}	3.41±0.03 ^{cd}	3.47±0.02 ^{cd}	3.38± 0.01^{d}	3.14±0.01 ^{e}	4.66± 0.06^{a}
21	3.46± 0.02^{c}	3.84±0.01 ^{a}	3.350.01± ^{d}	3.32±0.02 ^{e}	3.22±0.02 ^{f}	3.19±0.03 ^{f}	3.77± 0.05^{b}
28	3.37±0.04 ^{c}	3.80± 0.02^{a}	3.32±0.01 ^{c}	3.25±0.02 ^{d}	3.00± 0.01^{e}	2.97±0.01 ^{f}	3.67±0.01 ^{b}

Table 2b: Changes in pH of Fermented Milk Samples during Storage at Refrigerated Temperature

Storage days	AC	AR	ARC	BC	BR	BRC	AYBF
0	4.13 ± 0.02^{c}	5.49± 0.01^{a}	4.10± 0.01^{c}	4.03±0.03 ^{d}	4.34±0.02 ^{b}	3.75± 0.02^{e}	4.28±0.03 ^{b}
7	3.86 ±0.01^{c}	4.96 ±0.02^{a}	3.76 ±0.01^{d}	3.71 ±0.11^{e}	3.86 ±0.04^{c}	3.60 ±0.03^{f}	4.06 ± 0.03^{b}
14	3.85 ±0.05^{d}	4.70 ±0.01^{a}	4.11 ±0.02^{b}	3.70 ±0.01^{e}	3.71 ±0.02^{e}	3.33 ±0.03^{f}	4.01 ±0.01^{c}
21	3.85 ±0.02^{c}	4.33 ±0.02^{a}	3.87 ±0.01^{c}	3.67 ±0.03^{d}	3.52 ±0.02^{e}	3.58 ±0.02^{e}	4.25 ±0.03^{b}
28	3.67 ±0.01^{c}	4.03 ±0.03^{a}	3.60 ±0.02^{d}	3.58 ±0.05^{e}	3.41 ±0.02^{f}	3.45 ±0.01^{f}	3.86 ±0.01^{b}

Means with the same superscript within rows are not significantly different at P < 0.05

Keys : AC: Equal proportion of milk blends fermented with P. pentosaceus; AR: Equal proportion of milk blends fermented with L. paracasei; ARC: Equal proportion of milk blends fermented with combination of L. paracasei and P. pentosaceus; BC: Milk blend proportion 2:1:1 fermented with P. pentosaceus; BR: Milk blend proportion 2:1:1 fermented with L. paracasei; BRC: Milk blend proportion 2:1:1 fermented with combination of L. paracasei and P. pentosaceus; AYBF: 100% AYB milk fermented with commercial starter culture

3.2. pH and Acidity of fermented product

Reduction in pH of fermented milk samples, which follows the same trend as the microbial count as reported in Tables 1a and 1b, was observed during storage at both ambient and refrigerated temperatures (Table 2a and 2b). Initial pH of the products ranged from 3.75 to 5.49 before storage with sample AR having the highest value (5.49). Osman and Razig (2010) also reported a reduction in pH from 4.50 to 3.00 in soy yoghurt at the end of 20 days storage This finding is in agreement with observation in this study. A sharp decline in pH could be attributed to metabolism of the sugars present in the substrate to produce acids such as lactic acid and acetic acid which are major fermentation products of heterofermentative *Lactobacilli* and *Pediococcus* sp (Ebhodaghe *et al*., 2012). The rate of reduction in pH of fermented milk samples stored at refrigerated temperature was observed to be slower compared to samples stored at room temperature.

Microbial activities are slowed down at lower temperature, thereby affecting rate of substrate metabolism (Prescott *et al*., 1999). Change in titratable acidity of fermented milk samples at both ambient and refrigerated temperatures (Fig. 2a and 2b) increased during storage. Initial titratable acidity of the fermented products ranged from 0.53 to 1.02%. Titratable acidity of yoghurt from cow and soy milk composite was reported to be 0.15 - 0.33% (Olubamiwa and Kolapo, 2008). These values were lower compared to those obtained from the fermented milk samples in this study but lower than values (1.3 to 2.4%) reported by Adgidzi *et al.* (2011) for yoghurt-like product from tiger-nut. The result obtained in this study may be due to the initial starting premix which contains more fermentable sugars due to inclusion of coconut milk which may easily be converted into organic acids than in cow and soy milk. *Lactobacillus paracasei* produced the lowest amount of acidity during fermentation in the two milk blends used. Combination of both probiotic strains resulted in high acidity compared with product fermented with commercial starter culture.

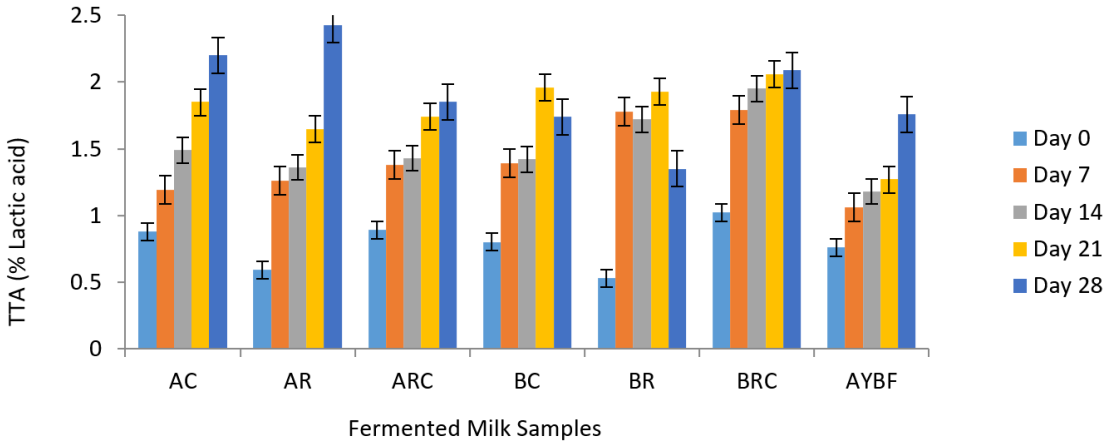

Figure 2a: Changes in Titratable acidity during Storage of Fermented Milk Samples at Ambient Temperature

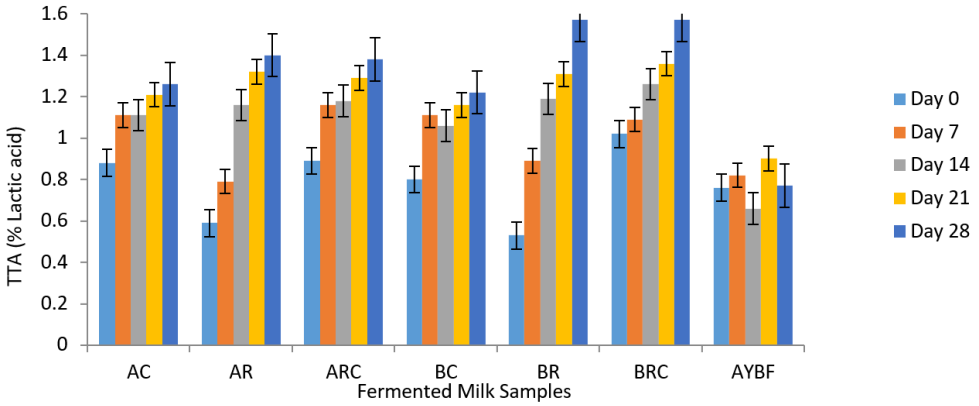

Figure 2b: Changes in Titratable acidity during Storage of Fermented Milk Samples at Refrigerated Temperature

Keys : AC: Equal proportion of milk blends fermented with P. pentosaceus; AR: Equal proportion of milk blends fermented with L. paracasei; ARC: Equal proportion of milk blends fermented with combination of L. paracasei and P. pentosaceus; BC: Milk blend proportion 2:1:1 fermented with P. pentosaceus; BR: Milk blend proportion 2:1:1 fermented with L. paracasei; BRC: Milk blend proportion 2:1:1 fermented with combination of L. paracasei and P. pentosaceus; AYBF: 100% AYB milk fermented with commercial starter culture.

Al-Kadamany *et al.* (2002) reported that lactic acid bacteria exhibited a substantial lag phase initially, with counts increasing thereafter and stabilized towards the end of storage. *Pediococcus pentosaceus* produces an equimolar mixture of acetate and lactate from pentose sugars (Wood and Holzapfel, 1995); this is probably the reason for high acidity observed in samples fermented with *P. pentosaceus* compared to the other probiotic strain. Steady increase was observed in titratable acidity of all fermented samples after 7 to 28 days storage in the refrigerator (0.77 -1.57) while a sharp increase was noted in samples stored at ambient temperature (1.35 - 2.43). Lower rate of metabolism in organisms at cold temperatures may be responsible for the difference in TTA of samples stored at the two temperatures. Fermentation of oligosaccharides (sugars) present in the milk samples by the LAB isolates to produce lactic acid bacteria was observed to increase at a very steady rate.

3.3. Total amino acid content of the product

Changes in amino acid content in fermented milk samples stored at the two storage temperatures are shown in Tables 3a and 3b. In freshly prepared fermented milk samples, total amino acid range was 4.27 -7.56 mg N/25 ml and after storage, it ranged between 4.20 and 10.50 mg N/25 ml in samples stored at ambient temperature while it ranged between 5.32 and 7.56 mg N/25ml in samples stored at refrigeration temperature. Milk from African yam bean and soybean are sources of protein which during fermentation and storage could be used by LAB for growth. LAB possess protease enzyme which metabolizes protein present in the fermenting medium into amino acids (Rani and Khetarpaul, 1999; Ebhodaghe *et al*., 2012).These amino acids may have been utilized by these microorganisms resulting in a decrease in amino acid content after 7 and 14 days of storage in some samples. As microbial cells enter the death phase, they could also contribute to the amino acid level of the fermented milk samples through autolysis of these cells.

Increase in amino acid content was observed in milk blend 1:1:1 fermented with *P. pentosaceus* (sample AC) (5.88 to 6.44 mg N/25ml), AR (7.14 to 7.56 mg N/25ml), ARC 4.64 to 5.04 mg N/25ml) and AYBF (4.27 to 5.32 mg N/25ml) stored at 4 °C and likewise similar trend was noticed in samples AC, ARC and AYBF at ambient temperature. This agreed with the findings of Olagunju and Ifesan (2013) who noted the increase of amino acid during fermentation of sesame seeds and Ebhodaghe *et al.* (2012) in storage of probiotic soymilk while decrease was observed in samples BC, BR and milk blend 2:1:1 fermented with combination of both strains (BRC) both at ambient and refrigerated temperature. Sample AC stored at ambient temperature had the highest content of amino acids after storage period; this could be a result of microbial death of the starter culture which greatly increased during storage and also protein metabolism releasing amino acids as end products. Germani *et al.* (2014) reported that in natural yoghurt a significant increase of the amount of free amino acids was observed during the period of shelf-life (97%) while in sweetened full fats and fruit yoghurt; instead, there is a lower increase of 33% and 39% respectively. This report justifies the observation in this study.

3.4. Reducing sugar content of the product

Observable changes in reducing sugar content of fermented milk samples during storage period at ambient and refrigerated temperatures are shown in Tables 4a and 4b. Fermentable sugar in the fermented samples ranged from 0.20 to 1.98 mg/ml before storage and varied between 0.00 and 0.99 mg/ml in samples kept at ambient temperature and 0.00 to 1.97 mg/ml in those kept at refrigerated temperature. Reducing sugar content was lowest in sample AYBF (0.20mg/ml). Inclusion of coconut milk in the milk blends could be the possible reason for high value of reducing sugar content in samples. Sample BR had the highest reducing sugar content (1.98 mg/ml). After 7 days of storage, there was reduction in the content of the reducing sugar of samples stored at ambient temperature but increase was observed in the sugar content of samples BC (1.03 to 1.24 mg/ml) and BRC (1.34 to 1.47 mg/ml) stored in the refrigerator. Generally, reducing sugar content of samples stored at ambient temperature decreased with time of storage and a total disappearance of sugar was noted in sample AYBF. The probability of the organisms utilizing the simple sugars for survival might be the reason for decrease in sugar content over the period of storage since high metabolic rate is observed at optimum temperatures.

At refrigerated temperature, reduction was gradual in some of the samples during storage; nevertheless, increment was noted in samples ARC (1.45 to 1.97 mg/ml), BC (1.03 to 1.45 mg/ml) and BRC (1.34 to 1.67 mg/ml) in line with the report of Ebhodaghe *et al.* (2012).

Table 3a: Total Amino Acids in Fermented Milk Samples during Storage at Ambient Temperature

Storage days	AC	AR	ARC	BC	BR	BRC	AYBF
0	5.88±0.24^{c}	7.14±0.14^{ab}	4.64±0.16^{d}	7.56±0.14^{a}	6.09±0.19^{c}	6.51±0.39^{bc}	4.27±0.07^{d}
7	5.67±0.07^{a}	4.34±0.42^{de}	3.64±0.28^{e}	4.90±0.14^{abcd}	5.18±0.17^{abcd}	5.08±0.42^{abcd}	5.18±0.42^{abcd}
14	5.18±0.14^{bc}	7.19±0.09^{a}	4.48±0.56^{cd}	6.86±0.42^{a}	3.64±0.00^{def}	5.15±0.07^{bc}	4.34±0.14^{cde}
21	7.50±0.42^{a}	5.74±0.14^{b}	6.86±0.14^{ab}	5.10±0.28^{b}	5.32±0.28^{b}	7.14±0.14^{ab}	7.00±0.56^{ab}
28	10.50±0.42^{a}	6.65±0.35^{cde}	7.42±0.42^{cd}	5.74±0.14^{efg}	4.20±0.28^{h}	6.30±0.70^{def}	9.10±0.42^{b}

Table 3b: Total Amino Acids in Fermented Milk Samples during Storage at Refrigerated Temperature

Storage days	AC	AR	ARC	BC	BR	BRC	AYBF
0	5.88±0.24^{c}	7.14±0.14^{ab}	4.64±0.16^{d}	7.56±0.14^{a}	6.09±0.19^{c}	6.51±0.39^{bc}	4.27±0.07^{d}
7	5.30±0.30^{abc}	5.04±0.28^{abcd}	5.39±0.21^{abc}	4.55±0.07^{bcd}	5.46±0.14^{ab}	4.48±0.32^{cde}	5.25±0.21^{abcd}
14	5.04±0.46^{bc}	5.40±0.28^{b}	5.18±0.32^{bc}	4.06±0.14^{def}	3.50±0.14^{ef}	5.18±0.42^{bc}	4.41±0.07^{cde}
21	5.32±0.01^{b}	6.16±0.28^{bc}	7.42±0.42^{a}	6.16±0.06^{bc}	5.60±0.56^{b}	5.28±0.14^{b}	5.18±0.14^{b}
28	6.44±0.0.11^{bc}	7.56±0.26^{a}	5.04±0.28^{g}	5.94±0.14^{d}	5.46±0.42^{d}	6.32±0.28^{cd}	5.32±0.28^{ef}

Total amino acids expressed in mg N/ 25mL

Means with the same superscript within rows are not significantly different at P < 0.05

Keys : AC: Equal proportion of milk blends fermented with P. pentosaceus; AR: Equal proportion of milk blends fermented with L. paracasei; ARC: Equal proportion of milk blends fermented with combination of L. paracasei and P. pentosaceus; BC: Milk blend proportion 2:1:1 fermented with P. pentosaceus; BR: Milk blend proportion 2:1:1 fermented with L. paracasei; BRC: Milk blend proportion 2:1:1 fermented with combination of L. paracasei and P. pentosaceus; AYBF: 100% AYB milk fermented with commercial starter culture

Table 4a: Reducing Sugar Content in Fermented Milk Samples during Storage at Ambient Temperature

Storage days	AC	AR	ARC	BC	BR	BRC	AYBF
0	1.12±0.04^{e}	1.23±0.01^{d}	1.45±0.04^{b}	1.03±0.01^{e}	1.98±0.02^{a}	1.34±0.04^{c}	0.20±0.02^{f}
7	0.89±0.10^{c}	0.23±0.01^{d}	1.10±0.06^{ab}	1.09±0.06^{ab}	0.88±0.04^{c}	1.15±0.05^{a}	0.06±0.02^{e}
14	0.70±0.06^{e}	0.18±0.00^{f}	1.32±0.06^{a}	1.13±0.01^{b}	0.93±0.03^{d}	1.08±0.02^{c}	0.08±0.02^{g}
21	0.48±0.07^{d}	0.27±0.02^{e}	1.08±0.03^{a}	0.88±0.14^{bc}	0.92±0.05^{bc}	0.67±0.15^{c}	0.00±0.00^{f}
28	0.29±0.01^{c}	0.22±0.02^{c}	0.87±0.07^{a}	0.65±0.03^{b}	0.91±0.05^{a}	0.99±0.01^{a}	0.00±0.00^{d}

Table 4b: Reducing Sugar Content in Fermented Milk Samples during Storage at Refrigerated Temperature

Storage days	AC	AR	ARC	BC	BR	BRC	AYBF
0	1.12±0.04^{e}	1.23±0.01^{d}	1.45±0.04^{b}	1.03±0.01^{e}	1.98±0.02^{a}	1.34±0.04^{c}	0.20±0.02^{f}
7	0.94±0.02^{d}	1.01±0.01^{cd}	1.40±0.10^{ab}	1.24±0.06^{b}	1.43±0.05^{a}	1.47±0.08^{a}	0.11±0.02^{e}
14	1.67±0.03^{b}	0.31±0.01^{e}	2.00±0.0.08^{a}	0.99±0.04^{d}	1.23±0.08^{c}	1.63±0.09^{b}	0.22±0.01^{e}
21	1.10±0.07^{bc}	0.76±0.05^{d}	1.24±0.09^{b}	1.74±0.03^{a}	1.05±0.16^{cd}	1.53±0.17^{a}	0.29±0.02^{e}
28	0.99±0.01^{d}	0.62±0.02^{e}	1.97±0.05^{a}	1.45±0.13^{c}	1.36±0.04^{c}	1.67±0.02^{b}	0.00±0.00^{f}

Reducing sugar expressed as mg/mL

Means with the same superscript within rows are not significantly different at P < 0.05

Keys : AC: Equal proportion of milk blends fermented with P. pentosaceus; AR: Equal proportion of milk blends fermented with L. paracasei; ARC: Equal proportion of milk blends fermented with combination of L. paracasei and P. pentosaceus; BC: Milk blend proportion 2:1:1 fermented with P. pentosaceus; BR: Milk blend proportion 2:1:1 fermented with L. paracasei; BRC: Milk blend proportion 2:1:1 fermented with combination of L. paracasei and P. pentosaceus; AYBF: 100% AYB milk fermented with commercial starter culture

Reducing sugar content was observed to be 0.00 mg/ml in sample AYBF after been stored for three weeks at refrigerated temperature unlike at ambient temperature with sugar disappearing after storage for 2 weeks.

4. CONCLUSION

Lactobacillus paracasei L27 and *P. pentosaceus* RS15 from local fermented foods were able to thrive in a non-dairy carrier, AYB-based fermented drink at both ambient and refrigerated temperature for 28 days and the physicochemical properties of these products compared well with that of fermented milk from soybean. In terms of probiotic potentials, sample BRC was the best product while it was rated as the second best with respect to sensory evaluation. The recommended shelflife of the beverage when stored below 4°C is 28 days and 21 days at 28°C.

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