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Biosynthesis of Enzymes in Fermented Foods by Lactic Acid Bacteria and Possible Use as Bio-Preservative in Fermented Milk

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Authors' contributions

This work was carried out in collaboration between all authors. Author SMA designed the study, performed the statistical analysis, wrote the protocol, and managed the first draft of the manuscript. Authors KOA, EDO and OEO were involved in the laboratory analysis, managed the literature searches used in the analyses of the study and the manuscript. Author OEO wrote the first draft. All authors read and approved the final manuscript.

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ABSTRACT

This work focused on isolating Lactic Acid Bacteria from fermented local milk, assaying the enzymes produced by the organisms, using them as starters in the fermentation of fresh milk and monitoring their bio-preservative activities.
Fresh milk samples pasteurized at 85°C for 15 min were inoculated with lactic cultures ($3x10^6$ cful/ml). Two organisms with high enzymatic activities were used as starters singly and in combination. LAB, yeast, coliform and aerobic count were monitored during fermentation at room temperature for five days.
Twenty four LAB were isolated from replicate samples of locally fermented milk called N*unu*. They

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were identified as *L. plantarum, L. fermentum, L. lactis, L.bulgaricus, L. casei and L. brevis.* The organisms were screened for the production of antimicrobials; hydrogen peroxide, diacetyl, lactic acid and Agar well diffusion method was used to monitor the antagonistic activities. These were used to screen the organisms and those with the best technological properties were selected as starters. Enzyme production and activities were monitored using Dinitrosalysilic acid method. Enzymes production; amylase, alpha-galactosidase, invertase and mellibiase ranged from 1.0-2.6 unit/ml from day 0-5 after which there was a significant reduction (0.5 unit/ml). Two organisms with high enzymatic activities; *Lactobacillus acidophilus and L. plantarum* were used as starters singly and in combination to inoculate the fresh milk samples which have been pasteurized earlier. The total LAB, yeast, coliform and aerobic count were monitored during fermentation at room temperature for five days. LAB count increased significantly from day 0-5 ($5.1x10^8$cfu/ml). A gradual increase was observed in yeast and aerobic count ($3.6x10^3$-$6.1x10^4$cfu/ml).
LAB demonstrated high enzymatic and bio-preservative activities on fresh milk by preserving the nutritional qualities of the milk and extending the shelf life of fresh milk. It also acts as natural preservative in milk by inhibiting spoilage microorganisms.

Keywords: Lactic acid bacteria; fermented and fresh milk; starters; enzymes; bio-preservatives; probiotics; shelf life.

1. INTRODUCTION

Milk could be defined as a highly nourishing and resourceful food [1]. It is obtained from the mammary glands of woman, and animals such as cows, dogs, goats and so on [2]. Milk being highly nutritious makes it possible for people to drink it raw or process it into a number of food products and such food products include; cream, butter, 'nunu', yoghurt, cheese, and ice cream [3].

In Nigeria, livestock rearing cores mainly on the production of cattle, sheep, goats, pigs and poultry indicating that Nigeria has a great potential in animal resources capable of meeting the nations dietary protein requirement. Milk obtained from its natural source is sterile however due to its high nutritional contents; it becomes a fertile ground for microbial inhabitation and is highly prone to contamination from external factors such as air, soil and milk handlers [4]. Milk is extremely resourceful therefore many other products can be derived from it and the ones that are of importance in this research is 'Nunu'; a naturally fermented milk product and 'Fura', which though is not a milk product is a semi-solid millet-based meal usually added to 'Nunu' before meal.

Even thou, in Food Microbiology, the presence of some pathogens or other microorganisms in food may cause food spoilage, however, microorganisms should not only be seen as an agent of food spoilage rather, some are capable of directly or indirectly producing enzymes and metabolites which are capable of preventing other spoilage organisms from proliferation thus preserving the food for some period of time [5].

Also, some group of enzymes such as Amylase, α- Galactosidase, Invertase, and Mellibiase could be produced by the lactic acid bacteria (LAB) present as natural flora in milk and milk products and these can play some note-worthy roles that can be of immense benefit in food digestion, industries and research establishments [6,7]. There is a need to carry out a study on this subject matter in other to increase the knowledge and understanding of them; this might further help in the area of applications.

2. MATERIALS AND METHODS

Collection of samples: Samples of 'fura de nunu' fermented local milk were purchased at five different intervals and different locations in Osun state for replicate study. Samples were then immediately taken to the laboratory for microbiological analysis.

Isolation of lactic acid bacteria: Serial dilutions of homogenised 'Fura de nunu' samples in 0.1% peptone saline were used for microbial isolation on MRS agar respectively. Plates were incubated for forty-eight hours at 30°C to isolate Mesophilic LAB [8]. The isolates were maintained on MRS agar plates containing 0.5 mg/ml of Nyastin kept at 4°C under anaerobic conditions [8,9].

Isolation of other organisms: Serial dilutions of samples were made. 1ml was taken from the tubes containing 10^{-1} and 10^{-3} by using a sterile Pasteur pipette and plated on Nutrient agar,

Potatoes Dextrose agar and MacConkey agar using the pour plate method [10].

Total microbial count: The number of colonies on each plate of media used was counted and recorded [10].

Identification of isolates from samples: Identification was based on growthon selective agar, colony morphology, Gram's reaction, biochemical test results and criteria for disregarding negative cultures. Results were analyzed using Bergey's manual, and other methods for the identification [11,12].

Inoculum preparation: The LAB isolates that were used for the various tests were prepared by inoculating a colony from a twenty-four hour old LAB streaked on a plate into a sterile 9 ml MRS broth. This was incubated for 24 h at 30°C [13].

Determination of inoculum size: The LAB isolates that were used were standardized according to Macfarland standard using $BaCl_2$ and HCl at the right proportion. The culture supernatant was also brought to the same optical density of 0.500 using sterile MRS broth [14].

Medium Preparation for enzyme assays: The Medium used was MRS-Starch broth in which the glucose has been substituted with equivalent amount (w/v) of soluble starch. The medium was dissolved and homogenized in a water bath (Uniscope 801A Model, England) after which it was dispensed into Enlenmeyer flasks in aliquots of 250 ml, plugged with non-absorbent cotton wool and Aluminum foil. It was sterilized at 121 ^{o}C for 15 min, allowed to cool. With a sterile pipette, 10 ml of the standardized isolates were inoculated into it. It was incubated at 30 ^{o}C on a shaker for 48 h.

Enzymes extraction: The culture-broth was centrifuged at 10,000 rpm for 15 min using Refrigerated centrifuge. The cell-free culture supernatant was labelled as the crude enzyme while the sediment (microbial cells) was stored in the refrigerator for further use. This was then assayed for amylase, Invertase and Mellibiase production and activity [15].

Amylase assay: Amylase activities of the organisms were determined using DNSA reagent method modified by [16]. 1 ml of culture supernatant was added to 1 ml of the substrate containing 1.2% (w/v) soluble starch in 0.1M phosphate buffer, pH 6.0. The enzyme-substrate mixture was incubated at 30°C for 10 min. The reaction was stopped by the addition of 5M NaOH. The amount of reducing sugar thus produced was estimated using 3, 5 – Dinitrosalicylic Acid (DNSA). 1ml of DNSA reagent was added to the filtrate-substrate reaction mixture above which then was heated in boiling water at 100°C for 10 min and cooled with distilled water. The absorbance was measured at 540 nm using Cecil 2031 automatics spectrophotometer.

1 ml of uninoculated blank similarly treated was used to set the spectrophotometer at zero [16].

Invertase assay: Invertase activity of the organisms was determined using DNSA reagent method modified by [16]. 1 ml of culture was added to 1 ml of a solution containing 1.2% sucrose in 0.1 M phosphate buffer, pH 6.0. The enzyme-substrate (sucrose) reaction mixture was incubated at 30°C for 10 min.

This was brought to halt by the addition of 5 ml NaOH. The amount of reducing sugar thus produced was estimated using 3,5-Dinitrosalicylic acid which was prepared. 1 ml of the DNSA reagent was then added to the filtrate-sucrose reaction mixture above and was heated in a boiling water bath at 100°C for 10 min. It was then cooled with distilled water. The absorbance was measured at 540 nm using Cecil 2031 automatic Spectrophotometer. 1 ml of un-inoculated blank similarly treated was used to set the spectrophotometer at zero. The amount of Invertase produced was read and recorded as being equivalent to the reducing sugar produced [17].

Melibiase assay: Mellibiase activities of the organisms were determined using DNSA reagent method;modified by [16]. 1 ml of culture supernatant was added to 1 ml of a solution containing 1.2% lactose in 0.1 m phosphate buffer, pH 6.0. The enzyme-substrate (lactose) reaction mixture was incubated at 30°C for min.

This was brought to a halt by the addition of 5M NaOH. The amount of reducing sugar thus produced was estimated using 3,5-Dinitrosalicylic acid which was prepared as stated in the appendix. 1 ml of the DNSA reagent was then added to the filtrate-sucrose reaction mixture above and was heated in a boiling water bath at 100°C for 10 min. It was then cooled with distilled water. The absorbance was measured at 540 nm using Cecil 2031 automatics

Spectrophotometer.1 ml of uninoculated blank similarly treated was used to set the Spectrophotometer at zero. The amount of Invertase produced was read separately and recorded while that of Mellibiase was read as being equivalent to the reducing sugar produced [17].

1 amylase unit is the amount of enzyme in 1 ml of the filtrate, which 1 mg of reducing sugar as glucose from a 1% starch in 1 hour at 30 ^{o}C. 1 Invertase unit is the amount of enzyme in 1 ml of the filtrate which releases 1 mg of reducing sugar, a glucose from a 1% sucrose in 1 h at 30^{o}C. 1 Mellibiase unit is the amount of enzyme in 1 ml of filtrate, which releases 1 mg of reducing sugar glucose as from a 1% lactose solution in 1h at 30°C.

Alpha (α) Galactosidase: The assay medium consisted of 200 µl of 100 mM sodium acetate buffer pH 5.0, 2.5 ml of 2 mM PNP – alpha Gal solution and 0.5ml of enzyme preparation. The assay was carried out for 15 min at 50°C and stopped by the addition of 1ml of 0.5 M sodium carbonate.

The amount of Para-Nitrophenyl-Beta-D-Galactopyraside (PNP-beta-G) released was determined at 410 nm by taking the absorbance. The undiluted mixture treated in the same way used to set the spectrophotometer to zero [18].

Inoculum preparation for bio-preservative purpose: *Lactobacillus acidophilus and L. Plantarum* were inoculated and incubated anaerobically at 30°C for 24 h separately in different tubes of MRS broth. The inoculums were made to McFarland standard and one ml of the broth culture was added to nine ml of pasteurized milk solution, mixed thoroughly and stored at room temperature. Control was also made, containing no Lactic acid bacteria [19].

Raw material: Fresh cow milk was obtained from the Teaching and Research Farm of Obafemi Awolowo University, Ile-Ifeand 200 ml of the sample was dissolved 1000 ml of distilled water to make a milk suspension. 10 ml each of sample were divided into bijou bottles and inoculated with 1ml each of *Lactobacillus acidophilus and L. plantarum* singly and in combination respectively [20].

Microbiological analysis: 1mlSamples were added to 9 ml of distilled water to make the initial dilution (10^{-1}). Serial dilutions up to 10^{-3} were then prepared and the colony forming units for Aerobes, Coliforms, Yeast and Lactic Acid Bacteria were followed and counted respectively on Nutrient agar, MacConckey agar, Potato Dextrose agar and MRS agar plates. The plates were incubated at 30°C for 48 h for Lactic Acid Bacteria, Nutrient agar, McConckey agar at 37°C for 24 h while the Potato Destrose agar plates were incubated at 25+/_ 2°Cfor two days [20].

3. RESULTS AND DISCUSSION

Amylase assay: For all the organisms, Amylase produced from starch hydrolysis at zero hour was very low. Amylase production increased at twenty-four hours with *Lactobacillus plantarum* and *Lactobacillus acidophilus* having the highest concentration of amylase. They all however produced larger amount of the enzyme at forty-eight hours indicating that all organisms could be capable of further amylase production.

With Invertase assay, *Lactobacillu splantarum* and *Lactobacillus acidophilus* gave similar concentration of enzyme produced as they hydrolyzed Sucrose. At zero hour, the enzyme production was low, this however increased at twenty-four hours with subsequently increase inconcentration at forty-eight hours. With *Lactobacillus lactis L pentosus and L. fermentum*, enzyme productions at twenty four hours and forty-eight hours were low with that of forty-eight hours being slightly higher than what was observed with the remaining organisms but with a drastic increase in production at forty-eight hours.

Table 1. Acidification of milk samples during fermentation

Milk Sample **1	Day 0	24hrs	48hrs	72 hrs	96 hrs
pH	6.0	5.8	5.5	5.0	4.2
Milk Sample** 2	6.0	5.7	5.4	5.4	4.3
Milk Sample ***3	5.7	5.5	5.0	4.5	4.0
4/0					

**1= Milk Sample fermented with L. plantarum*
***2= Milk Sample fermented with L. acidophilus*
****3= Milk Sample fermented with combined LAB Starter*

Table 2. Antimicrobial production by lactic acid bacteria and antagonistic activity against selected indicator organisms

Organisms	Hydrogen peroxide (g/L)	Diacety (g/L)l	Lactic acid (g/L)	Antagonistic activity against *S. aureus*	Antagonistic activity against *E. coli*
L. plantarum	0.0027±0.05[a]	0.2153±0.50[b]	0.4878±0.11[b]	+++	+++
L. lactis	0.0004±0.01[d]	0.0123±0.005[d]	0.1421±0.05[d]	-	-
L. pentosus	0.0004±0.01[d]	0.0171±0.010[e]	0.1134±0.05[d]	-	-
L. acidophilus	*0.0020±0.50[b]	0.2041±0.500[a]	0.5861±0.05[a]	+++	+++

** Values are Means of three replicate determinations ± Standard Error*

+ Values with the same superscript are not significantly different and values are compared within each column

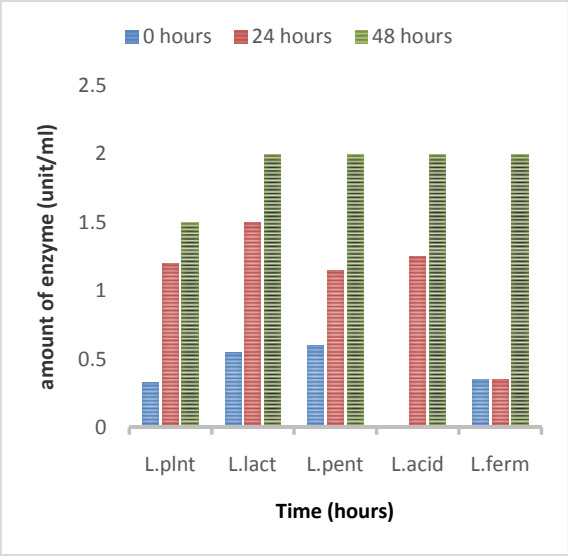

Fig.1. Amylase production (Unit/ml)

NB: L. plnt is Lactobacillus plantarum; L. lact is L lactis; L. pent is L. pentosus; L. acid is L. acidphilus; L. ferm is L. fermentum.

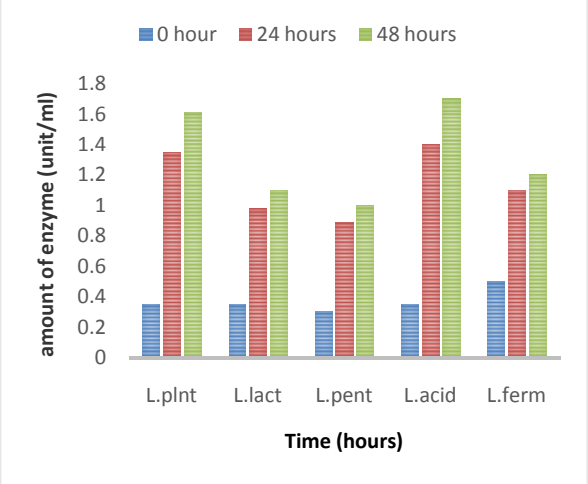

Fig. 2. Invertaseenzyme production (unit/ml)

For Melibiase assay: Same concentration of enzyme was produced by *Lactobacillus plantarum* and *Lactobacillus acidophilus* at zero hour with subsequent increase at twenty-four hours. This however increased drastically at forty-eight hours. *Lactobacillus lactis* and *Lactobacillus fermentum* showed almost similar trend but with variation in the actual concentration of produced enzyme. At forty-eight hours, the concentration increased to a maximum. Enzyme production by *Lactobacillus pentosus* was completely different from others; concentration increased from zero hour to twenty-four hours and later dropped to a much lower value at forty-eight hours.

α-Galactosidase assay: *Lactobacillu splantarum* rate of enzyme production increased in a step-wise manner with a remarkable enzyme production at forty-eight hours. *Lactobacillus lactis* and *Lactobacillus pentosus* enzyme production increased from twenty-four hours and the production later dropped at forty-eight hours with no further increase. For, alpha-galactosidase enzyme production, this was very low compared to others enzymes.

It however drastically increased through twenty-four hours to forty-eight hours in *Lactobacillus acidophilu* showerver producing the highest amount at twenty-four hours after which there was a further increase in enzyme production at forty eight hours. This result probably suggested that the organisms have reached its enzyme production limit and could not produce enzyme any longer. *Lactobacillus fermentum* followed the

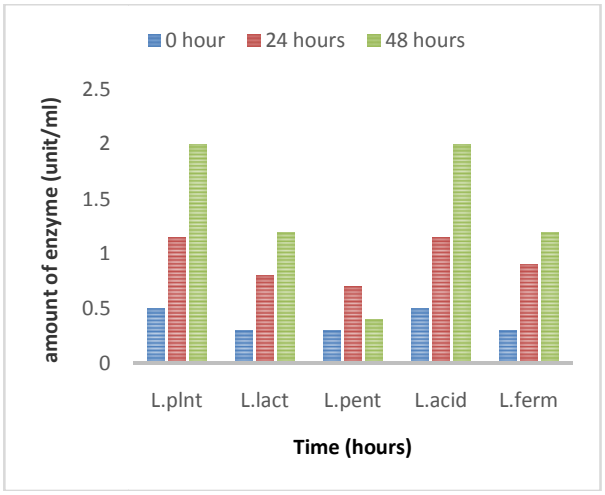

Fig. 3. Melibiaseenzyme production (unit/ml)

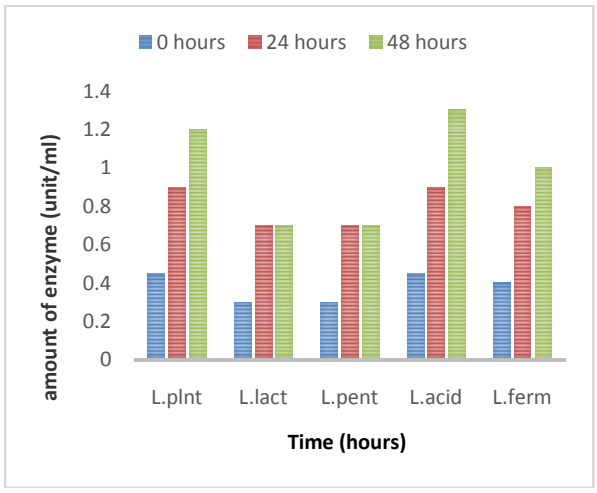

Fig. 4. Alpha-Galactosidaseenzyme production (Unit/ml)

same production trend as *Lactobacillus lactis* but the concentration of enzyme produced is not the same. Alpha- Galactosidase enzyme is useful in utilizing sugar in lactose for energy.

3.1 Bio-Preservative Effect

Bio-preservative effect of *Lactobacillus acidophilus,* an organism isolated from 'Fura de Nunu', was very remarkable; at zero hour, little growth was observed on Nutrient agar. The growth increased a bit at twenty-four hours and tremendously at forty-eight hours. With MacConkey agar, no growth was observed at twenty-four hours and no significant growth at forty-eight and ninety-six hours respectively.

The aerobic plate count increased significantly from 24 to 96 hr, no growth was observed on Mackonkey agar while the total coliform count was zero during the period. The total fungal count increased from 24 to 96 hrs. The total LAB count increased at forty-eight hours but to a level that was significantly higher at 96 hr.

With *L.plantarum*, the total aerobic plate count gave a remarkable growth at 24 hr. Growth reduced to a low level at forty eight hours and there was later a very high increase in growth at 96 hours. No growth was observed on MacConkey agar at twenty four and twenty-four hours respectively but at 96 hours there was a slight growth. With Potato Dextrose Medium, there was growth at 24 hr; further growth was observed at forty-eight hours. On MRS, there was growth at twenty four hours; there was increased growth at forty eight hours and further growth at 96 hrs.

The combined effect of the organisms exercised a further antagonistic effect on the organisms while LAB increased significantly from 24 to 96 hours. A reduction was observed in the total aerobic count and total yeast count when compared to the single starter. The combined activity of the starter caused a further decrease in the growth of the other flora of the milk apart from the starter.

3.2 Discussion

LAB play major a role in the overall acceptability and safety of fermented foods because their growth causes the reduction of the anti-nutrients, carbohydrate and pH of the foods due to lactic acid production [21]. This acidification process is one of the most desirable effects of the growth of LAB which is used in bio-preservation as reported by [22].

Fermented foods have been part of the human diet since the dawn of human civilisation, and have been used as a means of improving the shelf life, enhance palatability, safety, digestibility, and nutritional value of food for several centuries [23,24]. Lactic acid food fermentation is a process whereby microorganisms and their enzymes are used to

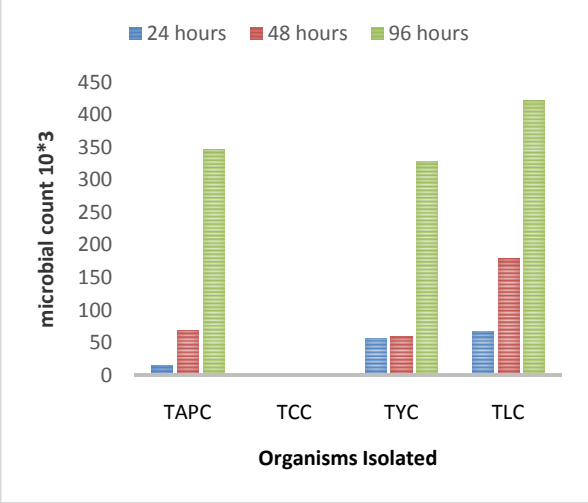

Fig. 5. Bio-preservative effect of *Lactobacillus acidophilus*

***KEY**: TAPC: Total Aerobic Plate Count, TCC: Total Coliform Count, TFC: Total Yeast Count, TLC: Total LAB Count*

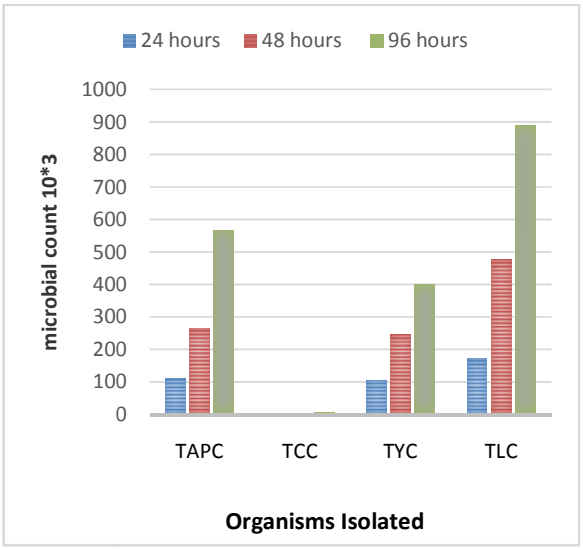

Fig. 6. Bio-preservative effect of *L. plantarum* on milk sample

***KEY**: TAPC: Total Aerobic Plate Count, TCC: Total Coliform Count, TFC: Total Yeast Count, TLC: Total LAB Count*

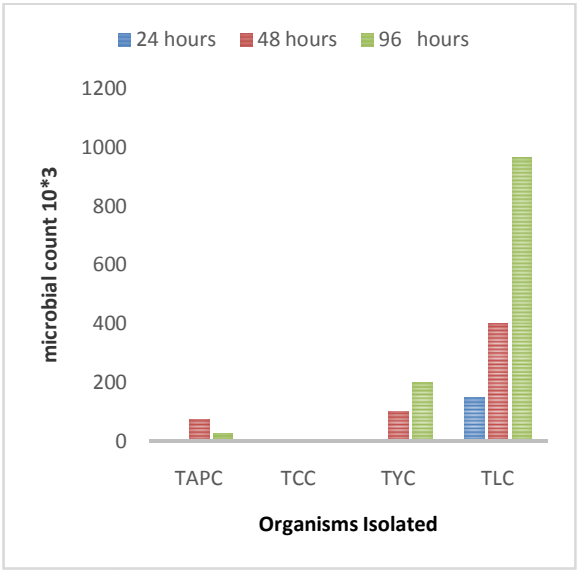

Fig. 7. Bio-preservative effect of combined starters

***KEY**: TAPC: Total Aerobic Plate Count, TCC: Total Coliform Count, TFC: Total Yeast Count, TLC: Total LAB Count*

convert fermentable sugars in the food substrate into mainly lactic acid and other by-products. Fermentation is widely used for food preservation in households and in food industries to develop a variety of food products with enhanced shelf life [25]. This food preservation technology is inexpensive; hence, it is of economic importance in developing countries. Microorganisms responsible for the fermentation of food products can be the microflora indigenously present on substrates, or they can be added as starter cultures after cooking or preparing the food or beverage.

Fermented milk has long been used as the main vehicles for probiotic strains. They have been used for incorporation of probiotic microorganisms and may offer a number of

advantages compared with naturally processed milk [26,22]. In addition to improving gut health, probiotics may play a beneficial role in several medical conditions, including lactose intolerance, cancer, allergies, hepatic disease, different gastrointestinal and urinary tract infections [27].

Enzymes are important in the various biological and biochemical processes that take place in the body. All the isolates produced the enzymes assayed for in varying concentration with *L. plantarum* and *L. acidophilus* producing the highest. This complies with the work of [28] who reported that *Lactobacillus spp*are able to hydrolyze starch and other sugars at concentrations into reducing sugars.

Furthermore, amylases are also produced by LAB. The degradation of starch and related polymers to yield products characteristics of individual amylolytic enzymes has been reported [28]. This property can be employed in food industries for which the amylase produced can be applied. This is in agreement with the work reported by [29].

The result obtained from the mellibiase activities by the Lactic acid bacteria isolates differ from one another; this observation might likely be due to the fact that the organisms produce the melibiase enzyme at varying degree. These strains can be used as starter culture with predictable characteristics and contribute to the development of small scale and commercial production of fermented foods with stable consistent quality. This is in accordance with the report of [14,30]. Invertase enzyme is useful in the breakdown of invert sugars to simple sugars. It is able to break down the lactose sugar in milk to simple sugars which can easily be absorbed by the body.

The ability of LAB to produce alpha-galactosidase which is very useful in digestion of different sugars. The importance of alpha-galactosidase produced by LAB ingested to overcome host deficiency of the enzyme. All LAB isolated in this study produce alpha-galactosidase enzyme in abundance, in which*L.plantarum*was the highest . This is also similar to an earlier report by [31] and [32] in production of enzymes by different species of LAB.

The lowering of the pH of fermented milk by LAB to below 4 through acid production inhibits the growth of pathogenic microorganisms which can cause food spoilage, food contamination and food poisoning. LAB because of their potential use as natural antimicrobial agents have been used to enhance the safety of food products. Most chemical preservatives used in processed foods have been found to contribute to health hazards among consumers when used in high doses [33]. Some preservatives have also been reported by Food and Drug Administration (FDA) to produce allergic reactions [33].

The increasing resistance of food spoilage microorganisms to current preservatives, the consumer's high demand for safe, minimally processed foods and the hazards associated with the use of high doses of chemical preservatives has led to the need for finding safer alternatives in food preservation. The application of LAB with the simultaneous control of factors that affect microbial growth can help to minimize food spoilage. The selection and addition of novel isolates of LAB may be the key to reducing the use of chemical preservatives, enhancing/improving nutrients and extending the shelf life of food products [34,35].

4. CONCLUSION

All organisms isolated in this study produced significant quantities of Amylase, alpha-galactosidase, Invertase and Mellibiase at the ninety-six hour; as a result, they would be most economically suitable at this period for the following purposes; in the digestion of insoluble starch in food industries and maltose production, it could also be useful in baking industry as an inclusion supplements in milk and milk products consumed by individuals suffering from lactose intolerance for complete metabolism of lactose sugar present in milk, . Also in the food industries; efficient ethanol could be manufactured and they can be included in animal feeds as digestion ingredients. Presence of reducing sugar is indicative of high food quality [36]. *Lactobacillus plantarum, Lactobacillus lactis*, *Lactobacillus acidophilus* and *Lactobacillus pentosus* would serve as very good and efficient sources of reducing sugar in the food industries.

LAB have been known to exercise a bio-preservative activity on other organisms by lowering the pH of the medium and producing metabolites which exhibit some antimicrobial properties especially in fermented foods [5]. This was observed by the fermentative activity of LAB during the fermentation of the milk in this study.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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