Determination of Survivability of Some Probiotic Lactic Acid Bacteria in Some Locally Produced Drinks

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Abstract

Foods are not only intended to placate hunger and supply essential nutrients but also to prevent the nutrition-related diseases and to improve physiological and mental wellbeing of consumers. This led to the development of probiotic foods. Probiotics are said to be live microorganisms which when adequately ($10^6/10^7$ CFU/mL) administered confer health benefits to the host. Probiotic drinks both dairy and non-dairy have been found to be carriers of these organisms to their site of action. This research aims at determining the survivability of probiotic lactic acid bacteria in some locally produced drinks. The isolation of the organisms from yoghurt and "nono" were conducted using cultural methods and further characterized using biochemical tests. Cell surface hydrophobicity (CSH), cellular auto-aggregation assays (CAA), hemolytic activities, bile salt and NaCl tolerance test were conducted to determine the probiotic properties of the organisms. The survivability of the organisms was evaluated after standardizing the inoculum using 0.5 McFarland standard and then serial dilution, plating and counting of visible colonies. Results were analyzed by ANOVA using SPSS software version 20.0. Out of the ten isolated and biochemically characterized lactic acid bacteria, three Lactobacillus species showed probiotic status, with CSH values of (79%, 75.2% and 91.3%) and CAA index of (79.3%, 74% and 83.5%), respectively, however there is no statistically significant difference between the CAA and CSH values of the isolates (p = 0.13 and p = 0.5, respectively), which indicates that all the isolates had approximately equal probiotic capabilities. "Kunun zaki" showed the highest survivability rate with the probiotic status being maintained for three days, and counts ranging from 1.4 to 1.05 x 10^6 CFU/mL; whereas "zobo" showed the least duration as a probiotic (one day, and a count of 1.16 x 10^6 CFU/mL. There is a significant difference (p=0.02) in the growth of the organisms over the incubation period (5 days), however, there is no significant difference (p=0.82) between the growth of the organisms growing on different drinks, indicating that time after incubation is the critical determinant of probiotic status, and that the isolates can grow equally well in all the drinks tested. This research reported the isolation of Lactobacillus species confirmed to be probiotics from yoghurt and "nono". Enumeration of probiotic LAB in all the drinks revealed the drinks were probiotic containing 10^6 CFU/mL for two (2) days with the exception of "Kunun zaki" that reached up to three (3) days as a probiotic drink. The pH level of all the drinks decreased and the drinks became acidic (pH range 6.0-1.8).
Keywords: Drinks, Lactobacillus species, Probiotics, Survivability.

INTRODUCTION

According to FAO/WHO, probiotics are live microorganisms that when properly provided, confer health advantages to the host (Vasie *et al.*, 2014) . The term ''probiotic'' is derived from a Greek word that means natural life and has a number of connotations over the years (Ukeyima *et al.*, 2010). Probiotics were first characterized by Lilly and Stillwell in 1965 as microorganisms that support the growth of other microbes (Ukeyima *et al.*, 2010). Among

other things, probiotics are thought to improve digestion, nutritional absorption, and food safety.

Probiotic bacteria are members of the Lactic Acid Bacteria (LAB) family, which also includes the lactobacillus species that are utilized in the production of fermented foods and convert carbohydrates into lactic acid and energy (Vasie *et al.*, 2014). When carbohydrates are fermented, lactic acid is the main by product produced by lactic acid bacteria, which are

Gram positive, non-spore forming, catalase negative, lacking in cytochromes, acid tolerant rods or cocci microorganisms that produce lactic acid as the major end product during fermentation of carbohydrates (Guesh *et al.*, 2019). Due to the rise in antibiotic resistance, there is focus in the use of *Lactobacillus* species as probiotics (Costa *et al.*, 2014).

Consumers no longer regard foods with respect only in terms of taste, flavor and immediate nutritional benefits, but also in terms of their capacity to offer beneficial health effects beyond their basic nutritional significance (Rahman *et al.*, 2015). Yoghurt and other dairy products are essential probiotic carriers (Costa *et al.*, 2014).

Yoghurt is a probiotic carrier and one of the earliest fermented milk products (Anika and Jayati, 2015). By fermenting milk with bacterial cultures made up of a mixture of *Streptococcus thermophillus* and *Lactobacillus bulgaricus,* yogurt is produced (Guesh *et al.,* 2019).

Locally made fermented yoghurt is produced, and consumption of these foods is customarily done. Since there is no standardization, it is manufactured by backslapping previously made yoghurt with fresh milk, which contains variety of bacteria. Additionally, the delay phase of the organisms causes the fermentation time in locally fermented yogurts to be prolonged, giving rise to a lenthy acidification coarse and making it difficult to generate an end product with a stable quality (Guesh *et al.*, 2019).

Additionally, probiotic yoghurts reduce lactose intolerance because lactase, an enzyme that convert lactose (milk sugar) in milk to lactic acid fermentation (Djomne *et al.*, 2011). Yoghurt is considered probiotic yoghurt if it has between 10^6 and 10^7CFU/mL at the time of consumption and 10^9CFU/mL at the time of manufacturing (Vasie *et al.*, 2014).
A beverage is a fluid that is intended for consumption by people (David *et al.*, 2020). It is a large and important subgroup of the food industry that includes many different types of thirst-quenchers and subgroups (Claudia *et al.,* 2019). Dairy and non-dairy beverages are also options (Craig and Fresan, 2021). Yoghurt and fermented milk drinks are examples of dairy beverages, whereas fruit, cereals, or vegetable based drinks like "Zobo", "Kunun aya" and "Kunun zaki" are examples of non-dairy beverages (Ndulaka *et al.*, 2018).

Regardless of socio-economic position, many households in Nigeria, especially in the North, consume "Zobo" one of the ready-to-eat healthy drinks (Sylvester *et al.*, 2015). The ingredient used to make "Zobo" drink is the dried calyces of the malvaceae family of *Hibiscus sabdariffa,* a hairy-branched aromatic schrub native to the tropical and semi-tropical areas of the world primarily in East Indies and West Africa. It is a nourishing beverage, and water is used to make it mostly (Osueke and Ehirim, 2004). Zobo" is medically known to lower blood pressure, cholesterol, and strengthens the immune system (Chibueze *et al.*, 2016).

"Kununaya" on the other hand is a drink native to the Northern Nigerian and is produced from a mixture of tiger-nuts, dates and coconut. It is a healthy beverage full of fibre and vitamin E as well as many other minerals. It is a non-alcoholic beverage that can be provided to guests as refreshment (Rowland *et al.*, 2021).

"Kununzaki" is a cereal based non-alcoholic fermented beverage which is consumed mainly in the Northern part of Nigeria. It can be produced from corn, sorghum, or millet (Ndulaka *et al.*, 2018). It has a creamy appearance, low viscosity, sweet bitter taste and a sprey, nutty flavor. It also contains essential vitamins and minerals (Bada *et al.*, 2018).

MATERIALS AND METHODS
Samples and Sample Collection
Three (3) samples each of both yohurt and "nono" were collected from different points (Kofar Durbi, KofarSauri, Kerau) and production companies (Freshyo, Hollandia, Mai-dabino) within Katsina for the research. They were collected in sterile leak proof plastic containers and transported in ice coolers to Department of Microbiology of Umaru Musa Yaradua University, Katsina for analysis (APHA, 2006).
Isolation of LAB from Yoghurt and Nono
De Man Ragosa and Sharpe (MRS) agar was used as suitable medium for isolation of lactic acid bacteria and was prepared according to the manufacturers' instruction. Discrete colonies were isolated using pour plate method. Ten fold serial dilutions using 1ml of the sample in 9ml of sterile distilled water was carried out up to the 10^{-5} dilution. One (1mL) of each suspension was separately introduced unto to two empty sterile petri dishes, MRS medium was then poured in and the plates were gently swirled to mix the sample and the medium. The plates were later incubated anaerobically at 37°C for 48-72 h for the growth of lactic acid bacteria. Discrete colonies were isolated and sub-cultured onto the same MRS medium to obtain pure isolates.

These pure isolates were then kept in a refrigerator at 4°C until required for further identification purposes (Michaylova *et al*., 2007). The morphological, biochemical and physiological identification methods used include Gram staining, endospore formation, Catalase, oxidase, indole, Citrate utilization, and MR-VP tests, as well as motility test, pH and NaCl tolerance tests, to identify lactic acid bacteria.

Determination of Probiotic Properties of the LAB Isolates

The probiotic characteristics of the isolates were identified using the following methods

Cellular Auto-aggregation Assay

The specific cell-cell interaction known as auto-aggregation was determined by the method described by Tochukwu *et al*. (2020). Overnight grown bacterial broth cultures were centrifuged at 12000 rpm for 5 min. to yield cell pellets. The pellets were repeatedly washed with Phosphate Buffer Saline (PBS) and re-suspended in 6 mL of PBS and the initial absorbance was measured at 600nm. The bacterial suspension was incubated at 37°C for 1 hour and the final absorbance of the supernatant was measured at 600nm. The percentage of cellular auto-aggregation was calculated by the formula:

Rate of Auto-aggregation % = OD initial – OD final / OD initial x 100

Assessment of Cell-surface Hydrophobicity

The ability of the bacterial cells to stick with hydrocarbons determines the extent of adhesion to the epithelial cells in the gastrointestinal tract is known as cell surface hydrophobicity. To determine cell surface hydrophobicity of the isolate, overnight grown bacterial broth cultures were centrifuged at 12000 rpm for 5 min. and the cell pellets were harvested. These cell pellets were then washed two times with phosphate buffer and re-suspended in 6 mL of phosphate buffer, followed by measuring the initial absorbance at 600 nm. After that 3 mL of the bacterial suspension was mixed with 2 mL of the hydrocarbon "xylene", and shaked well for 2 min.. The mixture was incubated and left undisturbed for 1 h for phase separation. The aqueous phase was then discarded carefully and the final absorbance was recorded at 600nm (Tochukwu *et al.*, 2020). The rate of cell surface hydrophobicity was calculated by the formula

Rate of Hydrophobicity % = OD initial – OD final / OD initial x 100

Bile Salt Tolerance Test

The test organisms were grown overnight in de Man Ragosa and Sharpe (MRS) broth. An aliquot of 0.1mL of the culture suspension was inoculated in tubes containing 10 mL of the MRS broth with 1.5 % bile. The inoculated tubes were incubated at 37ºC for 48 h. Presence of turbidity indicated a positive result and absence indicated a negative result (Succi *et al.*, 2005).

Hemolytic Activity

The method of Reuben *et al*. (2019*)* was used to determine the hemolytic activity of the isolates. Overnight grown cultures of the presumed LAB isolates in MRS broth were streaked onto blood agar and then incubated at 37ºC overnight. Hemolytic activities of the isolates were observed and recorded. The presence of Beta hemolysis was indicated by a clear colorless yellow zone surrounding the colonies depicting total lysis of the red blood cells. Alpha hemolysis was indicated by a small greenish zone to brownish discoloration of the medium depicting reduction of hemoglobin to methemoglobin which diffuses around, and Gamma hemolysis with no change observable change in the medium.

Determination of the Survival Activity of Probiotic Strain Isolated from Locally Fermented Yogurt in "Zobo," "Kunun aya" and" Kunun zaki"

After being streaked on MRS Agar, fresh cultures were cultured for 24h at 35ºC. The acquired culture was then diluted with water to achieve turbidity similar to tube 1 on the 0.5% MacFarland scale which is equivalent to 1.5 x 10^8 CFU/mL. The obtained culture was then injected into various drinks and incubated at 37ºC for 24hours before being kept in cold storage (Goncalves *et al.,* 2008). The serial dilution approach was used to count the number of viable cells. One (1mL) each dilution was taken out of the samples using sterile syringe, inoculated into petri plates, and then the prepared media was put on top and gently swirled. At 37ºC, the plates underwent anaerobic incubation for 24, 48, 72, 96 and 120 hours. Emergence of colonies were observed and recorded (Cheesbrough, 2006).

RESULTS

Characterization of Probiotic Bacteria from the LAB Isolates

The results of this study showed that based on the probiotic characterisation criteria, 3 out of the 10 Lactic Acid Bacteria (LAB) isolates were considered to be probiotic, as they were found to be non-haemolytic, and they showed positive tolerance to acidic pH (3-5), 1.5% bile salt concentration and 2-8% NaCl (Table 1). The isolates were all gamma haemolytic.

Statistically, the p value of cell surface hydrophobicity and cellular autoaggregation assay of the LAB isolates indicates no significant difference between the isolates (p = 0.13 and p = 0.5, respectively). This shows that all the isolates had approximately equal probiotic capabilities.

Table 1: Probiotic Properties of LAB Isolates

Probiotic Characterization	*Lactobacillus* spp 1	*Lactobacillus* spp 2	*Lactobacillus* spp 3
Cell Surface Hydrophobicity	79%	75.2%	91.3%
Cellular auto-aggregation assay	73.9%	74%	83.5%
Haemolytic activity	Gamma	Gamma	Gamma
NaCl tolerance			
2%	+	+	+
4%	+	+	+
8%	+	+	+
pH tolerance			
3	+	+	+
5	+	+	+
Bile salt tolerance test 1.5%	+	+	+

Key: + = Positive

Survivability of Probiotic LAB in Non-Dairy Drinks

The LAB counts of the survival of two (2) of the probiotic isolates in the various drinks for the period of 5 days are as follows.

Lactobacillus spp 1 survived in all the 3 drinks and showed probiotic status in "Kunun aya" for a period of 3 days, with LAB counts ranging from 1.53 x 10^6 – 1.14 x 10^6 CFU/ml. However the probiotic status in "Kunun zaki" was for a period of 2 days only, with counts ranging from 1.23 x 10^6 – 1.0 x 10^6 CFU/mL; and the probiotic status in "Zobo" drink was maintained for just one day, with a LAB count of 1.07 x 10^6 CFU/mL. There isa statistically significant difference (p= 0.02) in thegrowth of the organisms over the incubation period (5 days), however, there is no significant difference (p=0.82) between the survivability of the organisms in different drinks. This indicates that the time of incubation affects the probiotic status, and not the type of drink (Figure 1).
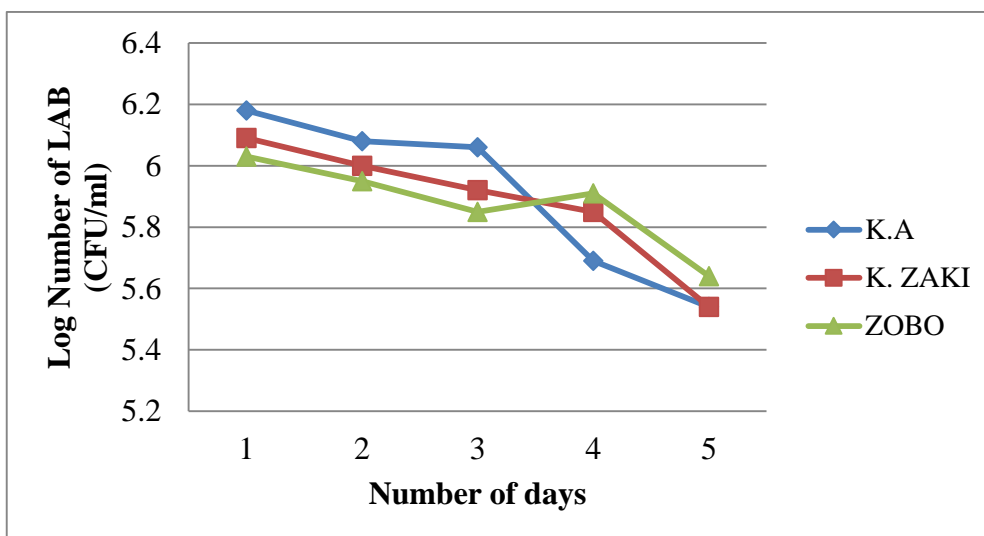

Figure 1: Survivability of Probiotic LAB in Non-Dairy Drinks (*Lactobacillus* spp1)
Key: K.A = Kunun Aya, K. Zaki = Kunun Zaki, Zobo = Zobo drink
Note: The P value for the growth of the isolates with regards to the incubation time (p = 0.023 < 0.05) indicated a significant difference between the organisms growing in the various drinks over the incubation period, while the P value for variation in growth between the substrates (p = 0.82 >

0.05) showed significant difference in the growth of the organisms in the drinks, over the incubation period.

Lactobacillus spp.2 also survived in all the 3 drinks however "Zobo" drink and "Kununaya" maintained the probiotic status for 1 day, with a colony count of 1.16 x 10^6CFU/mL and 1.05 x 10^6CFU/mL, respectively, whereas "Kununzaki" maintained the probiotic status for 3 days, with the survival of the isolates ranging from 1.4 x 10^6 - 1.05 x 10^6CFU/mL. There is a statistically significant difference (p= 0.001) in the growth of the organisms over the incubation period (5 days), but there is no significant difference (p=0.16) between the growth of the organisms on different substrates. The results were shown in figure 2.
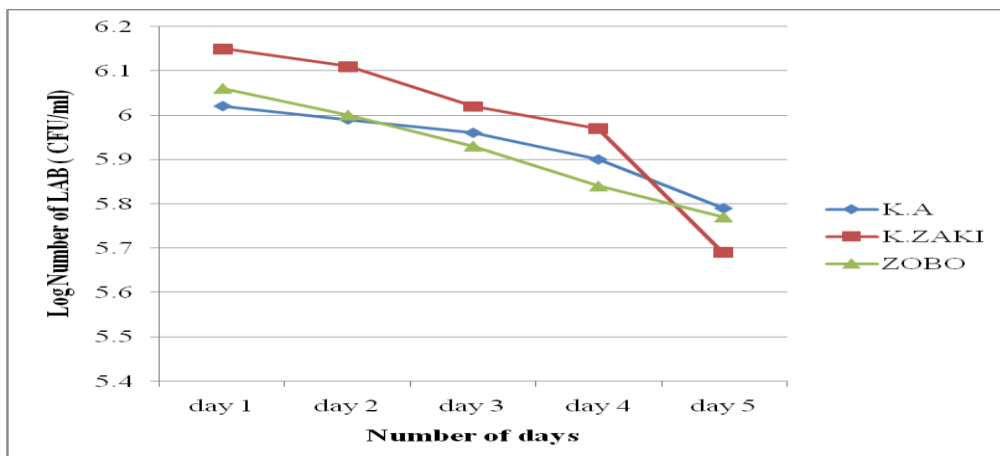

Figure 2: Survivability of Probiotic LAB in Non-Dairy Drinks (*Lactobacillus* spp 2)

Key: K.A = Kunun Aya, K. Zaki = Kunun Zaki, Zobo = Zobo drink
Note: The P value in for the growth of the isolates with regards to the incubation time is p = 0.001 < 0.05, showing a significant difference between the organisms growing in the various drinks over the incubation period; while the P value for variation in growth between the substrates is (p = 0.16 >0.05), showing no significant difference in the survivability of the organisms growing in the various drinks over the incubation period.

The pH Level Produced by *Lactobacillus* species

The change in pH level by *Lactobacillus* specie1 in the drinks for the period of 5 days is as follows: "Kunun aya" (5.7 – 2.83), "Kunun zaki" (5.84 – 2.24) and "Zobo" drink (4.00-1.53), whereas the pH level produced by *Lactobacillus* specie2 is as follows: "Kunun aya" (5.7 – 2.75), "Kunun zaki" (5.84 – 3.55), "Zobo" drink (4.00 – 2.48).There is a statistically significant difference (p <0.05) in the pH level over the incubation period (5 days) and also between the types of drinks. The results were shown in figures 3 and 4 respectively.
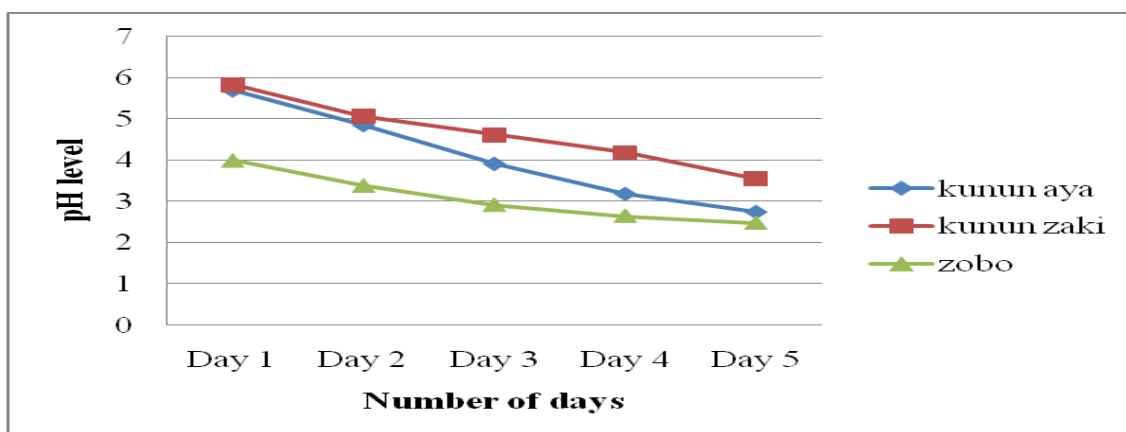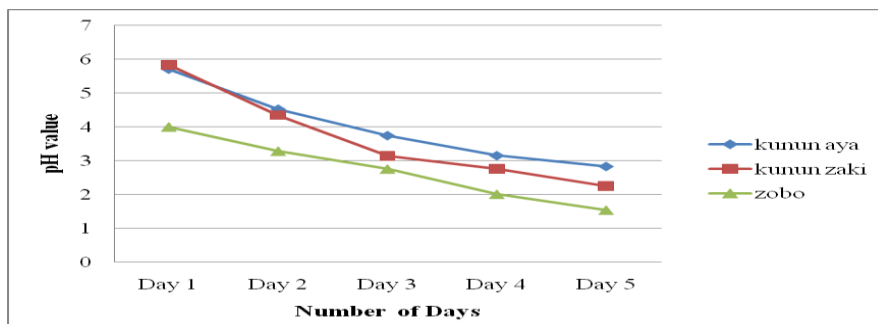
Figure 3: pH of the Drinks Produced by *Lactobacillus*specie 1 over 5 days Incubation Period
Note: The P values (0.008 and 0.0002) indicated significant differences in the pH production with regards to both the incubation timeand the type of drink, respectively, which shows that incubation time and drink type significantly influence the lactic acid content of the drinks over the incubation period.

Figure 4: pH of the Drinks Produced by *Lactobacillus* specie 2 over 5 days Incubation Period
NOTE: The P value for difference in pH production with regards to both substrate (type of drink) and incubation time was 0.0002, which shows that both substrate type and incubation time significantly affect the lactic acid content of the drinks.

DISCUSSION

The probiotic characteristics, including cell surface hydrophobicity (75.2-91.3%) and cell auto-aggregation assay (73.9-83.5%) values obtained in this study agreed with what was reported by Miao *et al*. (2020), who reported *Lactobacillus salivarus* as non-haemolytic LAB with cell surface hydrophobicity of 34.49% and cellular auto-aggregation assay value of 95.6%. Rakesh *et al*., (2019) on the other hand reported *Lactobacillus* spp.asnon-haemolytic LAB with cell surface hydrophobicity ranging from 50.3% to 77.8%, and cellular auto-aggregation assay values of 51.02% to 78.95%. Moreover, Rine *et al*. (2019) reported *Lactobacillus reuterii* as probiotic bacteria with cell surface hydrophobicity of 40 – 70%, and cellular auto-aggregation assay values of

37.66% - 59.5%. The high value of cellular auto-aggregation assay is important for the colonization, kin, kind recognition and survival of the bacteria on the surfaces of the interacting cells (El-Shama*et al*., 2021), while the high value of the cell hydrophobicity will facilitate the adhesion of the microorganisms to biotic and abiotic factors (Carina and Arturo, 2021).

The ability of probiotic *Lactobacillus* species to grow in non-dairy beverages had been reported by previous workssuch as the work of Yusnita *et al*. (2017), where the growth of *Lactobacillus plantarum* in roselle beverage was shown over a period 20 days and was able to maintain its probiotic status in the beverage for the whole period (20 days). Additionally non-dairy drinks are not usually considered as a major source of

calories nevertheless, fruits, vegetables and cereals serve as an ideal drink for the development of probiotic beverages and offer several advantages for the growth and survival of probiotic organisms (Panagiotis*et al*., 2016). The present research also agreed with the work of Yusnita *et al*.(2017) with regards to the increase in the pH content of roselle beverage, however, they reportedno statistically significant difference in the pH content over the incubation period. In contrast, this study found out the pH (lactic acid production) showed statistically significant variation with the incubation time. This work also agreed with the work of Omole and Oranusi (2019) who reported that the pH of wine produced from *Hibiscus sabdariffa* using *Lactobacillus fermentum* ranged from 3.47 – 2.76,over the period of 24hours.

CONCLUSION

Conclusively, this research reported the isolation of *Lactobacillus* species from yoghurt and "nono", which were confirmed to be probiotics. Enumeration of probiotic LAB in all the drinks revealed that the drinks were probiotic, containing 10^6CFU/mL for two (2) days, with the exception of "Kunun zaki" that reached up to three (3) days as a probiotic drink. The pH level of all the drinks decreased and the drinks became acidic (pH range 6.0-1.8).

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