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Viability of probiotic lactic acid bacteria in Malay apple (*Syzygium malaccense*) during fermentation and storage

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Abstract

For lactic acid bacteria (LAB) to be considered probiotic it must colonize and survive the gut environment, also for maximum health benefits it must be ingested in a sufficient amount. Hence, this study was aimed at investigating the probiotic potential of lactic acid bacteria and their viability in Malay apple juice. Five LAB of yoghurt origin were obtained and screened for probiotic potential using acidic pH (1.5) tolerance, bile salt (0.3 %) resistance and non-hemolytic activity as the selection and safety criteria. The compositional and physicochemical characteristics of the apple juice during fermentation were assayed using standard methods. The viable cell counts were determined using standard plate count method. Of the 5 LAB isolates 2 identified as members of *Lactobacillus* and *Lactococcus* genera showed good probiotic characteristics with acidic pH and bile salt survival rate of above 80 %, also the 2 LAB isolates were non-hemolytic. After the 72 h fermentation increase in protein content ranged from 0.53 to 1.21 %, titratable acidity increase ranged from 0.35 to 0.53 % and a reduction in pH ranged from 4.54 to 3.58 were obtained, also the viable cell counts were at 4.12±0.38, 2.35±0.15 and 3.18±0.10 CFU/ml in the *Lactobacillus, Lactococcus* and mixed culture fermented juice respectively. At the end of the 30 days cold (4^{o}C) storage the viable cell counts were >106 CFU/ml. The Lactobacillus fermented juice had more overall acceptability (7.7) by the panelists. In conclusion the studied LAB isolates were viable probiotics and Malay apple juice a suitable carrier for probiotics.

Keywords: Probiotic, Lactic acid bacteria, Viability, Malay apple.

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INTRODUCTION

Lactic acid bacteria (LAB) are heterogeneous group of bacteria that produce lactic acid as the primary end product during carbohydrate fermentation (Naeem *et al*., 2012). Lactic acid bacteria consist of so many genera with members of *Lactobacillus*, *Lactococcus*, *Streptococcus*, *Pediococcus*, *Enterococcus*, *Leuconostoc, Streptococcus, Weissella* as the most

encounteredin food and food products (Leong *et al*., 2014; Naeem *et al*., 2012). Lactic acid bacteria are commonly used as starter culture in the fermentation of most dairy products (milk, yoghurt, cheese) (Bintisi and Anthanasoulas, 2015; Bintsis, 2018). The intolerance of so many people to dairy products has driven interest of researchers to production of nondairy fermented alternatives with vegetables and fruits. Lactic acid bacteria have been successfully used in the technological transformation of so many fruits and vegetables as reported by some literatures such Yuasa *et al*. (2020) on citrus juice, Wang *et al*. (2022) on kiwifruit juice and Kwaw*et al*. (2018) on mulberry.

Probiotics are life microorganisms considered as prospective functional food which when ingested in sufficient quantity alters the gut microbes of consumers (FAO/WHO, 2001), which in turn improves the health.Probiotics can play both functional and technological role in food products. For a microorganism to be considered a probiotic it is crucial that it survives and colonizes the gastrointestinal environment(Gu *et al*., 2008; Olatunde *et al*., 2018; Somashekaraiah*et al*., 2019). To efficiently benefit consumers, probiotics must be viable in food at a recommended minimum level of 10^6 CFU/ml (Moreno *et al*., 2006; Karimi and Cruz, 2011). The viability of probiotics in products is affected by food matrices, production and storage conditions (Karimi and Cruz, 2011).

Malay apple which is a pink colored tropical fruit with white pulp widely consume in the tropical region (Oliveira *et al*., 2011) is technologically underutilized despite its potential. Previous studies on Malay apple have shown that the various parts of the plant (fruit, stems, seeds and leaves) are potential antioxidants and contain bioactive compounds (Batista, *et al*., 2017; Nune *et al*., 2016). Although, Hosein *et al*. (2015) produced wine with malay apple fruit, this plant still remains understudied and underutilized.

The fact that most fruits especially of the apple categories are deficient in amino acids and free peptides which help in maintaining the balance of the microorganisms found in the gut as stated by Yang *et al*. (2022), and also the moderate minerals and vitamins content of malay apple, and their lower calories compared to that of"fuji" apple or the "gala" apple necessitates addition of value to malay apple juice product through fermentation technology for market penetration as functional food. Hence, this study was aimed at investigating the probiotic potential of lactic acid bacteria from yoghurt and their viability in Malay apple juice as probiotic delivery medium.

MATERIALS AND METHODS

Assay for potential and safe probiotic isolates

Lactic acid bacteria

Five lactic acid bacteria (LAB) isolates of yoghurt origin were collected from microbiology laboratory at Michael Okpara University of Agriculture, Umudike, Abia State. They were maintained in MRS (de Man, Rogosa and Sharpe) agar (HiMedia, Mumbai, India) stabs as pure cultures. The LAB isolates were screened for their probiotic potentials and hemolytic activities. The safe and potent probiotic LAB isolate's identities were reconfirmed based on their biochemical and physiological characteristics with some tests such as catalase, Gram staining, spore staining, motility, gas production from glucoses and carbohydrate utilization using the following sugars; ribose, xylose, mannitol, lactose (HiMidia, Mumbai, India), obtained results were compared with existing information in Boone *et al*. (2001).

Selection of acid and bile salt-tolerant isolates

The method of Tambekar and Bhutada (2010) with slight modification was adopted in screening the LAB isolates for acid and bile salt tolerance. For acid tolerance, 5-M HCl was used to adjust MRS broth to pH 1.5 and pH 6.0 which was used as control, 100 µl of activated cultures were inoculated separately into the pH adjusted broth and incubated anaerobically at 30 °C for 4 h. Using pour plate method, 1 ml of each 4 h broth culture diluted to 10^{-3} using peptone water was inoculated on MRS agar plates and then incubated at 30 °C for 48 h under anaerobic conditions. Plates were observed for growth to identify acid tolerant isolates and colonies were counted to determine survival rate. For bile salt tolerance, the acid-tolerant isolates were selected and further assayed for bile salt tolerance using the same protocol as described for acid tolerance. Here the MRS broth was supplemented with 0.3 w/v bile salt (Himedia, Mumbai, India), a broth without bile salt served as the control.

Hemolytic Activity

The hemolytic activity of the LAB isolates was determined using the procedure described by Yadav *et al*. (2016). The potential probiotic isolates were inoculated onto blood agar plates containing 5% (w/v) sheep blood using streak method, plates were incubated at 37 ^{o}C . After 48 h incubation, the plates were observed for non-hemolytic, α-hemolytic (greenish halo) and β-hemolytic (clear halo) activities.

Sample collection and preparation of substrate

Sample of well ripened locally available malay apples were purchased from Orieugba market in Umuahia, Abia State. The fruits were sorted by removing diseased, green, and damaged apples, the apples were thoroughly washed to remove soil and other impurities under a running tap water, air dried and blanched in water bath at 60 ^{o}C for 20 min. The fruits were deseeded and juices extracted using a juice extractor and further filtered through a muslin cloth with a sieve (0.8 to 1.1 mm pore size) to get a clear juice. Apple juice was sterilized at 110°C for 10 min and cooled to room temperature before fermentation.

Inoculum preparation and fermentation of the malay apple juices

The LAB isolates with probiotic potentials were activated by successively subculturing them twice in MRS broth cultures at 30 ^{o}C for 24 h. Inoculum was prepared by harvesting cells at 10,000 g from a 24 h culture, then washed with sterile normal saline. The cells were diluted till an initial viable count of about 10^{6} CFU/ml was obtained. The prepared Malay apple juices (100 ml) were transferred into 250 ml Erlenmeyer flasks juice were sterilized at 110°C for 10 min and cooled to room temperature before fermentation. All samples except from the control were inoculated with 5 % of the prepared inoculum, for the mixed culture a ratio of 1:1 was used, fermentation was carried out at 30 °C for a period of 72 h. Fermented juices were stored immediately at 4^{o}C for 30 days. During fermentation and storage samples were collected at 0, 24, 48, and 72 hours and during storage at day 10, 20 and 30 respectively for further analysis.

Proximate and physicochemical analysis

The furnace incineration gravimetric method was used for ash content determination, fat content was estimated by the continuous solvent extraction method, protein content was determined by the kjeldahl method, moisture content by gravimetric method and the carbohydrate content determined by arithmetic difference method (AOAC, 2005). Total Titratable acidity, expressed as per cent lactic acid, was determined by titration against 0.1N NaOH using phenolphthalein as an end point indicator. The pH value was obtained by using a digital pH meter after standardizing it with buffers of pH 4.0 and 9.015.

Microbial analysis of probiotic malay apple juice

The standard plate count method was used to determine the viable count of microbes in the fermented juice. Lactic acid bacterial counts were determined using MRS (de Man-Rogosa-Sharpe) agar (Himedia. Mumbai, India) supplemented with 0.14 % sorbic acid and pH adjusted to 5.7 using 10% HCl. The plates were incubated at 30 ^{o}C for 24 hours (Balestra and Misaghi, 1997), all results were expressed as CFU/ml juice.

Sensory evaluation

A 10-member panel evaluated the sensory characteristics of the fermented fruit juices. The 9-point Hedonic scale with corresponding descriptive term ranging from 1 (dislike extremely) to 9 (extremely like) was used to determine the taste, color, flavor, texture and overall acceptability (Ranganna, 2005).

Statistical analysis All the experiments were carried out in triplicates, and subjected to ANOVA (analysis of variance) results were expressed as mean value ± standard deviation.Significance was defined at $p< 0.05$, and means were separated using Duncan's test.

RESULTS

Probiotic potential and safety evaluation of isolates

Of the five lactic acid bacterial isolates obtained from the Laboratory, two of these isolates (LAB2 and LAB5) showed high tolerance to acidic pH (1.5) with survival rate of 89.2 % and 87.2 %

respectively and high bile salt (0.3%) tolerance with survival rate of 91.7 % and 88.6 % respectively (Figure 1). The two isolates also had no hemolytic activities as there was no clear zone in the plates. The LAB were morphologically and biochemically confirmed to be members of two genera *Lactobacillus* (LAB2) (Gram positive short rod, non-motile, none spore former, catalase negative, produced gas in glucose and could utilized ribose, mannitol, xylose and lactose) and *Lactococcus* (LAB5) (Gram positive coccoid, none motile, none spore former, catalase negative, could utilize ribose, mannitol, lactose but unable to ferment xylose and produce gas in glucose) (Boone *et al*., 2001).

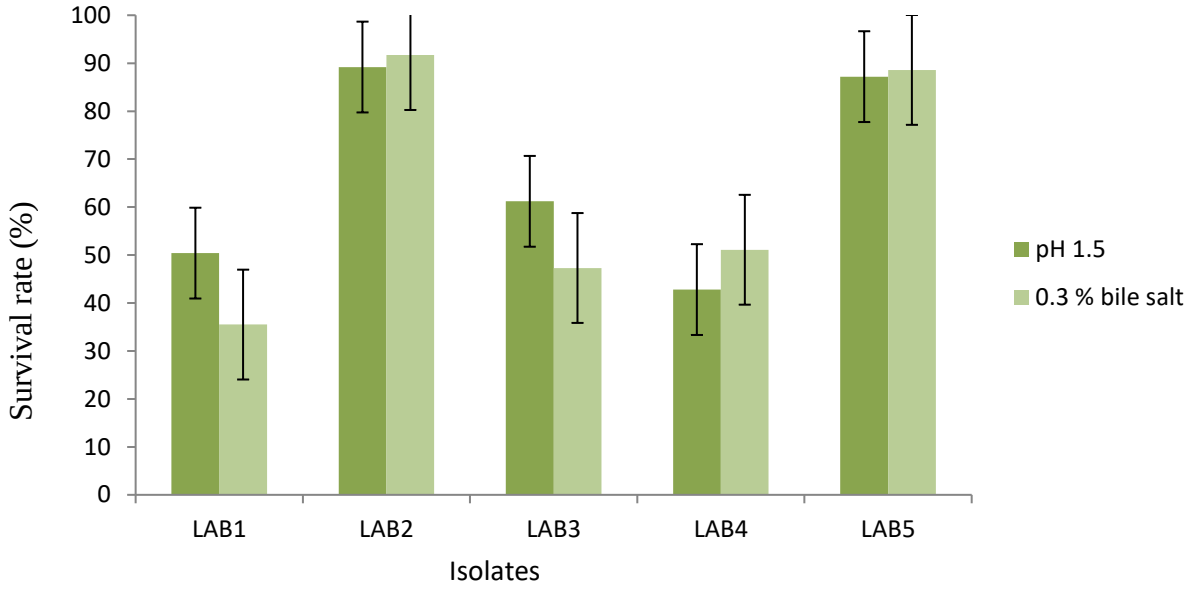

Figure 1: survival rate count of LAB under acidic pH and 0.3 % bile salt concentration.

Proximate composition of the probiotic malay apple juice

As shown in Table 1, the proximate compositional properties of the lactic acid bacterial fermented juice were significantly higher than the control. There was a significant increase in protein (1.21±0.01%) and ash content (0.42±0.02%) with mixed culture fermentation (*Lactobacillus* and *Lactococcus*). Also a significant (p< 0.05) lower carbohydrate content of 10.6±0.01 % was obtained with the mixed culture. There was no significant difference (p<0.05) in the moisture content obtained from the samples.

Physiochemical properties of the fermented malay apple juice samples

The physiochemical properties results obtained (Table 2), there was a reduction in pH value as the fermentation time increased. In the contrary, the titratable acidity of the malay apple juice samples increased as the fermentation time increased and this inversely affected the pH. The sample D (un-inoculated control) recorded the least pH of 3.58±0.13, and highest titratable acidity of 0.53±0.04 at 72 hours fermentation.

Table 1: Proximate composition (%) of the probiotic malay apple juice

Sample	Protein (%)	Ash (%)	Moisture Content (%)	CHO Content (%)	Lipid (%)
A	0.96±0.14^{b}	0.33±0.00^{c}	86.3±0.41^{a}	11.8±0.14^{c}	0.12±0.00^{c}
B	0.76±0.12^{c}	0.38±0.00^{b}	86.8±0.70^{a}	13.7±0.03^{b}	0.13±0.00^{b}

C	1.21±0.01[a]	0.31±0.02[d]	86.7±0.14[a]	10.6±0.01[d]	0.10±0.14[d]
D	0.53±0.70[d]	0.42±0.02[a]	86.5±0.70[a]	16.8±0.70[a]	0.16±0.02[a]

Key: A= Sample with *Lactobacillus* sp.**,** B= Sample with *Lactococcus* sp.**,** C = sample with mixed culture A and B (1:1)**,** D = Sample without LAB. Results are expressed as mean ± standard deviation from the three replicates. Values with different superscript letters in the same column indicate a significant difference *(p*<0.05).

Table 2: Physiochemical properties of the fermented Malay apple juice samples.

	24h		48h		72h	
Sample	pH	TTA (%)	pH	TTA (%)	pH	TTA (%)
A	5.00±0.15[a]	0.15±0.01[c]	4.88±0.07[a]	0.23±0.08[d]	4.54±0.10[a]	0.35±0.02[c]
B	5.02±0.11[a]	0.24±0.01[b]	4.86±0.19[a]	0.29±0.00[c]	4.42±0.12[b]	0.39±0.09[b]
C	4.91±0.05[a]	0.31±0.04[a]	4.50±0.25[b]	0.37±0.01[b]	4.01±0.16[c]	0.42±0.01[b]
D	4.72±0.21[b]	0.33±0.02[a]	4.39±0.09[c]	0.42±0.11[a]	3.58±0.13[d]	0.53±0.04[a]

Key: A= Sample with *Lactobacillus* sp.**,** B= Sample with *Lactococcus* sp.**,** C = sample with mixed culture A and B (1:1)**,** D = Sample without LAB. Results are expressed as mean ± standard deviation from the three replicates. Values with different superscript letters in the same column indicate a significant difference *(p*<0.05).

Lactic acid bacteria viability during fermentation and cold storage

The LAB count during fermentation and storage as presented in Table 3, showed that the initial viable count at 0 h (< 1 h) fermentation decreased compared to the initial inoculum count. An increase in viable cell count was recorded for all culture from 24 h to 48 h, afterward a decline in viable count was observed at 72 h fermentation. The highest count was observed from the *Lactobacillus* sp. sample at 5.27±0.77 x 10^7CFU/ml followed by the mixed culture fermented juice at 4.10±0.11 x 10^7CFU/ml after 48 h fermentation. At cold storage (4^{o}C) there was a continuous decline in viable cell count but a count of above 10^6 CFU/ml for the inoculated samples was still maintained after 30 days cold storage.

As presented in Figure 2, *Lactobacillus* sp. fermented juice was the preferred with a general acceptability of 7.7 followed by the *Lactococcus* sp. fermented juice with acceptability of 7.2, the least preferred was the control (5.5 acceptibility) as judged by the panelists.

DISCUSSION

Selection of a safe LAB with probiotic potential is as important as selection of a suitable substrate during fermentation. To maximize the health benefits of LAB consumption it should be able to survive the extreme environment of the gastrointestinal tract. Out of the 5 LAB isolates obtained 2 (LAB2 and LAB5) exhibited probiotic potentials through their high survival rate in an acidic pH (1.5) and a 0.3 % bile salt concentration for 4 h. This depicts the capability of these isolates to survive gastric juice low pH and the small intestine condition (Gu *et al*., 2008). Olatunde *et al*. (2018) and Somashekaraiah *et al*. (2019) reported LAB with high survival rate under low pH and bile salt (0.3 %) which are in agreement with this report. The absence of hemolytic activities of the isolates confirmed that they are safe to be used as starter culture in fermentation (Singal *et al*., 2019). The identity of the isolates conformed to the presumptive characteristic of LAB as Gram positive, catalase negative and none spore formers, while further biochemical characteristics of the isolates identified them to be of *Lactobacillus* and *Lactococccus* genera (Boone *et al*., 2001).

Table 3: Viable counts (CFU**/ml) of lactic acid bacteria in Malay apple juice during fermentation and storage**

Treatment	Fermentation 30^{o}C				Storage 4^{o}C		
	0 hour (10^5)	24 hours(10^7)	48 hours (10^7)	72 hours (10^7)	Day 10 (10^7)	Day 20 (10^7)	Day 30 (10^7)
A	8.2±1.00[aa]	3.79±0.22[ba]	5.27±0.77[ca]	4.12±0.38[da]	3.71±1.00[ea]	3.62±0.21[fa]	2.78±0.12[ga]

B	7.5±1.01^{ab}	2.82±0.23^{bc}	3.92±0.98^{cc}	2.35±0.15^{dc}	2.15±0.07^{ec}	1.70±0.07^{fc}	1.11±0.89^{gc}
C	5.2±1.2^{ac}	3.26±0.78^{bb}	4.10±0.11^{cb}	3.81±0.10^{db}	3.27±0.50^{eb}	2.06±0.15^{fb}	1.77±0.20^{gb}
D	ND	1.38±0.27^{bd}	1.63±0.75^{cd}	1.10±0.91^{dd}	0.98±0.11^{ed}	0.87±0.00^{fd}	0.01±0.05^{gd}

Key: A= Sample with *Lactobacillus* sp.**,** B= Sample with *Lactococcus* sp.**,** C = sample with mixed culture A and B (1:1)**,** D = Sample without LAB. ND= no significant growth. Results are expressed as mean ± standard deviation from the three replicates. Values with different superscript letters in the same column indicate a significant difference (*p*<0.05).

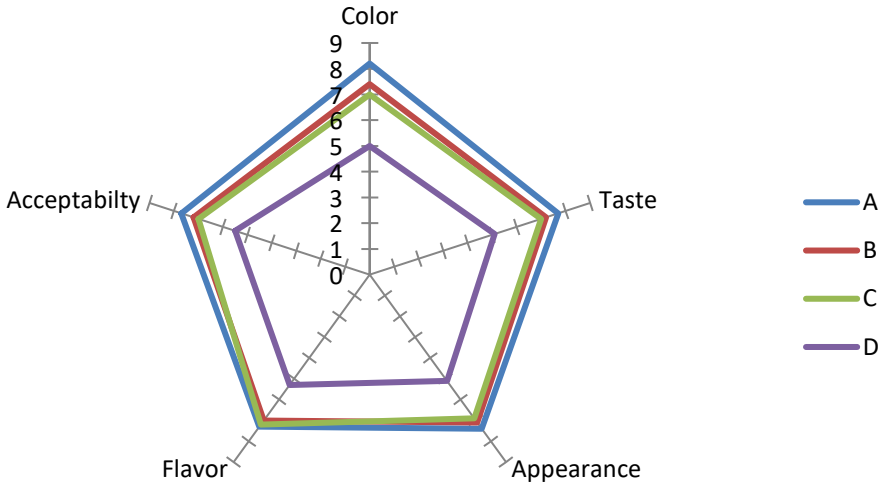

Figure 2: Organoleptic characteristics of probiotic malay apple beverage
Key: A= Sample with *Lactobacillus* sp.**,** B= Sample with *Lactococcus* sp.**,** C = sample with mixed culture A and B (1:1)**,** D = Sample without LAB

Protein content was higher in the samples inoculated with LAB than in the un-inoculated samples, Mousavi *et al*. (2011) attributed this to the lactic acid bacteria secretion of extracellular protein. The least ash content was recorded in samples inoculated with mixed culture (0.31±0.02 %), and the highest was from the un-inoculated control (0.42±0.02 %) this might have resulted from the metabolic activities of the LAB as stated by Kaprasob *et al*. (2017). The moisture content varied slightly across the samples but not significantly (*p*<0.05), the slight variation might be as a result of insignificant evaporation as the fermentation took place under same condition. The carbohydrate content was highest in the un-inoculated control 16.8±0.70 % and lowest in the mixed culture samples 10.6±0.01 %. This might be attributed to the fact that carbohydrate is essential for growth and metabolism in lactic acid bacteria, studies by Pimentel *et al*. (2015), Sivudu *et al*. (2014) and Tahmasebia *et al*. (2019) agreed with this. Comparing the lipid content result of the un-inoculated control (0.16±0.02%) which was higher than that of the inoculated samples one can say that lactic acid bacteria metabolized the lipid. The fact that lactic acid bacteria can produce extracellular lipases which possibly hydrolyzed the lipids into readily utilizable forms explained the decline in lipid content with LAB inoculation.

The physicochemical properties of the malay apple juice during the 72 h fermentation showed a continuous decline in pH and increase in titratable acidy for all the sample treatments.

However, a higher titratable acidity and lower pH value was recorded in the control sample, probably because the control was fermented by autochthonous lactic acid producing bacteria, which might have higher capability of producing organic acids. The accumulation organic acid (lactic acid) during the carbohydrate fermentation resulted to increase in acidity hence a decrease in pH and increase in titratable acidity (Pimentel *et al*., 2015). Similar result was obtained by Jafar *et al*. (2019) and Yang *et al*. (2022).

The viable cell population reflects the growth status during fermentation and cell stability during cold storage. During the 72 h fermentation the initial cell count recorded at 0 h (< 24 h) was below the initial inoculum count (10^6 CFU/ml), this might be as a result of environmental induced stress as the pre-culture medium (MRS broth) condition is different from that of the fermentation medium (Malay apple juice) (Reddy *et al*., 2015;Yanez *et al*., 2008). Rapid increase in LAB count was recorded from the 24 h to 48 h fermentation, and a decline in viable cell count was obtained at 72 h fermentation, this might have resulted from accumulation of metabolites and reduction in pH (Kyung *et al*., 2006). *Lactobacillus* sp. grew more rapidly to a viable cell population of 5.27±1.77 CFU/ml at 48 h fermentation. The least LAB population growth was recorded with the mixed culture during fermentation, the interaction between the two species might have affected the cell growth as some probiotics can produce bacteriocin against other probiotics (Kumar *et al*., 2015). During the 30 days cold storage, there was a continuous decline in viable cell population of all the lactic acid bacteria cultures. However, all the inoculated samples had a viable cell population above 10^6 CFU/ml which is the threshold levelof probiotic for maximum health benefit (Moreno *et al*., 2006;; Mousavi *et al*., 2011; Karimi and Cruz, 2011).

The sensory evaluation results indicated that the inoculated samples were more acceptable than the un-inoculated control. The low acceptance of the un-inoculated juice by the panelist might be attributed to the fact the juice was fermented spontaneously by unselected autochthonous lactic acid producing bacteria which might haveproduced high quantity of LAB by products (lactic acid, acetaldehyde and diacetyl) hence the drawbacks. Generally the probiotic juice produced with *Lactobacillus* sp. was more preferred this result is in line with the investigations of Jafar*, et al*. (2019), Reddy *et al*. (2015) and Shaikh *et al*. (2019).

CONCLUSION

In this study 2 isolates of lactic acid bacteria from yoghurt belonging to two genera *Lactobacillus* and *Lactococcus* survived the gastrointestinal tract simulated conditions of acidic pH (1.5) and 0.3 % bile salt, with a survival rate of above 80 %. The LAB isolates can be regarded as safe, as they showed no hemolytic activity on blood agar. The 2 LAB isolates were satisfactorily viable as they had counts >10^6 CFU/ml the recommended minimum therapeutic level in malay apple juice after 30 days cold (4^{o}C) storage. In addition, all the probiotic fermented juice had overall acceptability of 7.0 and above. In conclusion, the 2 studied LAB isolates exhibited potent probiotic characteristics and malay apple showed that it can be an excellent delivery medium for probiotics. Hence, it is recommended that lone underutilized fruits found especially in the tropics should be processed to functionalized product to improve food security.

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