ISSN 2989-0705 (Online)|Vol 1|Issue 1|July 2023

Journal of Applied Learning | BMS make learning happen

Determination of the Acid Tolerance in *Lactobacillus* Isolated from Yogurt

Singappuly Arachchillage Senurie Maria Seneviratne,[1] Punsisi Rasara Weerasooriya[1*] and Daniel George[1]

[1]School of Science, Business Management School (BMS), Sri Lanka

*punsisi@bms.ac.lk

Abstract

Probiotics, classified as live microorganisms, are incorporated into a variety of dairy/non-dairy products such as yogurt/beer to confer a wide range of health benefits to consumers upon the consumption of adequate quantities. *Lactobacillus* is one of the most abundantly utilized genera of probiotics in food fermentation and the most commonly found bacterium in yogurt. The research study was aimed to isolate and identify *Lactobacillus* from five commercial brands of yogurt purchased from the local market and to determine its' acid tolerance. Bacteria were isolated by culturing the samples on MRS agar using quadrant streaking. Morphological characterization and four biochemical tests (Gram's staining, acid-fast staining, endospore staining and catalase test) were carried out to distinguish if the isolated bacteria were *Lactobacillus* before the identified colony was sub-cultured in nutrient broth. The acid tolerance assay was performed at pH 7.2 and 3 at 0 hours and after a 3-hour incubation period using spectrophotometry. Acid tolerance was statistically analyzed using one-way ANOVA, using SPSS statistics software. Morphological characteristics and the biochemical test results revealed that the bacteria were Gram-positive, non-acid-fast, vegetative cells and catalase-negative supporting the presence of *Lactobacillus*. No significant difference was observed according to the p-value which indicated the ability of *Lactobacillus* to tolerate acids to a certain extent. This study aided the understanding that the *Lactobacillus* species utilized by manufacturers were beneficial acid tolerant probiotics which could render the desired health benefits to the consumers.

Keywords: *Lactobacillus*, probiotics, yogurt, acid tolerance

1. Introduction

Probiotics, derived from a Greek word which means "for life", are classified as "live microbial feed supplements" that provide a beneficial effect to the host when consumed, by improving the gut microbiota.[1,2,3] Under the genera of *Lactobacillus* and *Bifidobacterium*, *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus plantarum*, *Bifidobacterium lactis*, *Bifidobacterium longum*, and *Bifidobacterium bifidum* are the species of beneficial probiotic bacteria.[4] Probiotics are widely found in dairy products (cheese, yogurt, milk, buttermilk, ice-cream) and in certain non-dairy products (beer, olives, pickles, cereals and chocolate).[1,2] Probiotics render several beneficial and desirable properties as demonstrated in figure 1.0. The variety of properties impart numerous health benefits to humans as mentioned by Shi *et al*[4] such as ameliorating dermal/oral health, mitigation of postmenopausal disorders, exerting antihypertensive effects.

Lactobacilli are lactic acid bacteria which are Gram-positive, non-motile, non-spore-forming coccobacilli or rods. *Lactobacillus* is catalase-negative and has a G+C content which is generally below 50 mol%. Furthermore, they are aciduric or acidophilic, aerotolerant or anaerobic and strictly fermentative (homofermentative/ heterofermentative). Lactobacilli have convoluted nutritional requirements which include vitamins, carbohydrates, salts etc.[5,6] They have an optimum pH and temperature in the range of 5.5–6.2 and 30–40 °C respectively for the effective growth of the bacteria[7]. Genus *Lactobacillus*, phylum *Firmicutes*, class *Bacilli*, order *Lactobacillales*, and family *Lactobacillaceae* present the taxonomic classification of *Lactobacillus*.[8] Lactobacilli are

employed in biotechnology, pharmaceuticals, feed and food fermentation as a silage inoculant, vaccine carrier, probiotic and dairy starter.[5]

Yogurt is derived from the Turkish word "ya-urt" which means "sour milk".[9] Yogurt is a semisolid dairy product produced by the lactic acid fermentation of milk. Yogurt provides innumerable health benefits and has a high nutritive value as it provides an exceptional source of calcium, vitamin D, amino acids, potassium, riboflavin, vitamin B6/B12.[10-13] Depending on the chemical composition of yogurt, they have been classified based on the fat content into non-fat, low-fat and regular yogurt.[12] Yogurt is commonly produced with cow's milk, however, goat's milk, camel's milk, soy milk, corn milk, oat-based milk, coconut and tigernut milk may also be used to manufacture various yogurt types.[11] According to Banerjee *et al*[12], both two different groups of yogurt contain *Lactobacillus*. *Lactobacillus bulgaricus* in standard culture yogurt and *Lactobacillus acidophilus* in Bio- or probiotic yogurt. This makes yogurt an excellent and ideal source to be used to extract *Lactobacillus* for research.

Lactobacilli exhibit probiotic properties such as antioxidant activity, antimicrobial activity against pathogenic species, bile and acid tolerance and adhesion to internal surfaces.[14,15] Acid tolerance is the ability to withstand/avoid the destructive effects of an acidic environment such as in the stomach. Lactobacilli have adopted several acid tolerance mechanisms to protect themselves from gastric acids as it enables them to grow and multiply. Acid tolerance is one of the crucial criteria used to select potential probiotics.[16] F1-F0-ATPase, malolactic fermentation, ADS/ADI, AA decarboxylation (GAD system), biofilm and cell density, metabolic regulations, protection and repair of cellular macromolecules are the mechanisms utilized by *Lactobacillus* in acid tolerance.[17,18]

The F1-F0-ATPase is a proton pump that pumps out excessive H+ ions from the cytoplasm to the external environment by PMF, consuming ATP to increase the intracellular pH to promote bacterial cell survival.[19,20] Lorca and Valdez[21] justified through their research that *Lactobacillus acidophilus* CRL 639 indeed utilizes the F1-F0-ATPase as a mechanism of acid tolerance when under acidic stress. In the ADI system, ArcD initially transports arginine into the cell, which is converted to citrulline, and ammonia catalyzed by ADI. Citrulline is phosphorolysed into ornithine and carbamoyl phosphate catalyzed by OTC. Ornithine is exported out of the bacterial cell while carbamoyl phosphate and ADP are converted to ammonia, CO_2 and ATP, catalyzed by CK. Ammonia neutralizes the intracellular H+ ions whereas the ATP is made available for the F1-F0-ATPase[22]. Guo *et al* [16] confirmed the involvement of the ADI system as an acid-tolerant mechanism in *Lactobacillus plantarum* ZDY 2013 in their research findings. In the GAD system, glutamate is transported into the cells via GadC and is converted to GABA utilizing the excessive intracellular H+ ions and is catalyzed by glutamate decarboxylase. This raises the pH in the cytoplasm thus resisting the acidic stress in *Lactobacillus brevis* and *Lactobacillus acidophilus NCFM* as mentioned in the study conducted by Wang *et al*[23] and Lyu *et al*[24].

The main objective of this study was to determine the acid tolerance of *Lactobacillus* in yogurt. It is important to understand the acid tolerance ability of *Lactobacillus* which determines their ability to withstand the HCl acid in the stomach to survive and multiply, and hence provide the desired health benefits to the consumers. These study findings will be useful to educate manufacturers that using *Lactobacillus* with acid tolerance property is extremely important and beneficial as a probiotic in yogurt to elevate the overall product quality.

2. Methodology

2.1 Sample collection/preparation. Five yoghurt samples were purchased from the local market and stored at 4℃ in a refrigerator. The samples were labelled A-E respectively. Approximately 2 g of each yoghurt was homogenized in 100 ml beakers with 500 µL of autoclaved distilled water.

2.2 Isolation of Lactobacillus. A loopful of each homogenized sample was cultured on De Man, Rogosa and Sharpe (MRS) agar (with amphotericin B) using quadrant-streaking and incubated at 37°C for 48 hours.

2.3 Gram's staining. A loopful of the isolated bacterial colony from each culture was mixed with a water droplet to make a thin smear. The smear was heat fixed and flooded with crystal violet for 60 seconds, Gram's iodine solution for 60 seconds, Gram's decolorizer for 5 seconds and finally with safranin for 50 seconds. The smear was rinsed with distilled water after each step. The slide was blot dried with a tissue paper and observed under 100X oil immersion on a compound light microscope.

2.4 Acid-fast staining. A thin smear was made on each glass slide as mentioned in Gram's staining. The smears were air-dried and heat fixed. The smears were flooded with carbol fuchsin and heated until vapor rose. The slides were rinsed with distilled water. The smears were flooded with acid-decolorizer for 15 seconds and methylene blue for 60 seconds. The smears were rinsed with distilled water after each step. The slides were blot dried with a tissue paper and observed under 100X oil immersion on a compound light microscope.

2.5 Endospore staining. A thin smear was made on each glass slide as mentioned in Gram's staining. The smears were air-dried and heat fixed. Small pieces of Whatman filter paper were placed on each smear. Malachite green was added to the filter papers and the staining rack was placed over a water bath of 100°C. Filter papers were kept moist by adding drops of malachite green. Afterwards, the slides were allowed to cool down, filter papers were removed and the smears were rinsed with distilled water. The smears were flooded with safranin and rinsed with distilled water after 40 seconds. The slides were blot dried with a tissue paper and observed under 100X oil immersion on a compound light microscope.

2.6 Catalase test. A thin smear was made on each glass slide with the inoculation loop using a loopful of the isolated colony from each culture. The slides were placed over black background. 3% H_2O_2 was added to each smear and observed for effervescence.

2.7 Sub-culturing. A loopful of the isolated *Lactobacillus* colony from each culture was sub-cultured in 20 ml of nutrient broth in a falcon tube and incubated at 37°C for 48 hours. The subcultures were then stored at 4°C in the cold conditions.

2.8 Acid-tolerance assay. This method was modified from Sahadeva *et al*[25]. 5 ml of each subculture was centrifuged for 3 minutes at 4000 rpm. The supernatant was discarded. 5 ml of peptone water was added to each pellet and shaken. 2 ml of each sample solution was mixed with 0.5 µL of 37% HCl to adjust the sample pH to pH 3. pH 7.2 was used as the control. Three absorbance readings were obtained at 0 hours and 3 hour incubation at room temperature for both pH 7.2 and 3 at 600 nm wave length.

2.9 Statistical/data analysis. The acid tolerance ability was statistically analyzed using one-way ANOVA, using SPSS statistics software. ($P < 0.05$ were regarded as statistically significant).

3. Results

3.1 Cultures and colony morphology. Isolation and morphological characterization of potential lactobacilli cultures were plated on MRS agar

after an incubatory period of 48 hours at 37°C (figure 1).

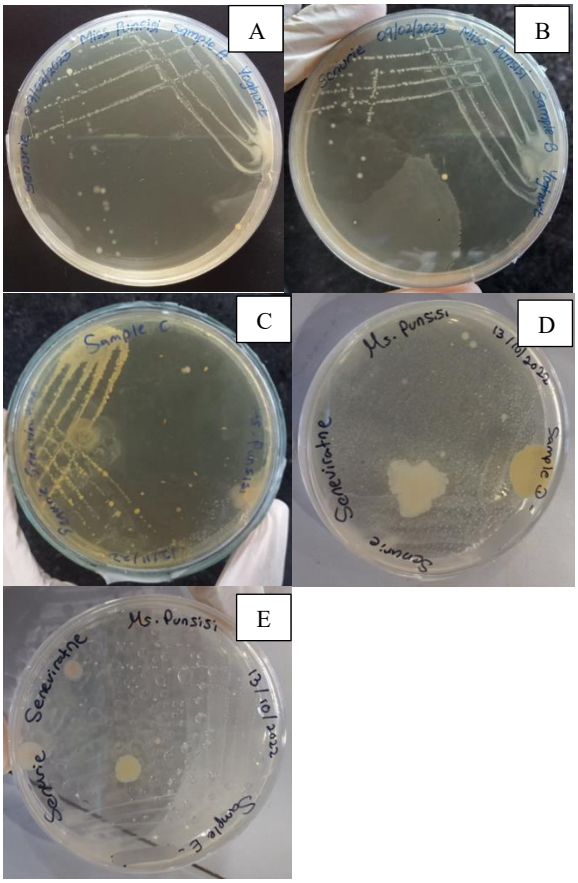


Figure 1. Colony morphology of yogurt sample cultures A-E on MRS agar after an incubation period of 48 hours at 37 °C.

The morphological characteristics of the colonies from each sample were observed to be opaque, smooth and creamy/milky white in colour, with a circular form, convex elevation and entire margin. A fungal growth was observed in culture D and E.

3.2 Gram's staining. Microscopic observation of the isolated colonies after Gram's staining were obtained (figure 2). Rod-shaped/Bacillus bacteria were observed in all the samples A-E. The isolated bacteria were observed in purple colour after the Gram's staining.

3.3 Acid-fast staining. Microscopic observations of the isolated colonies after acid-fast staining were given in figure 3. monolayer of rod-shaped bacteria was stained blue in samples A-E.

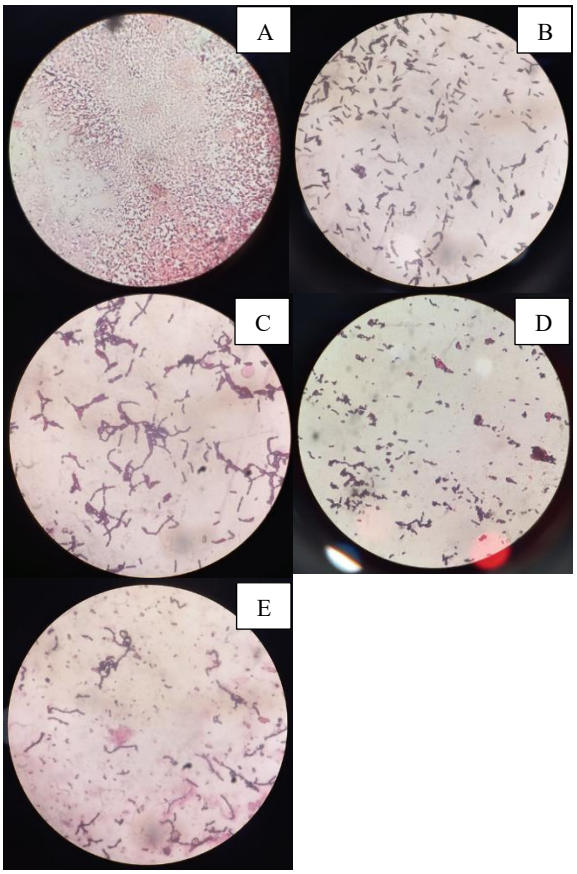


Figure 2. The Gram's stains of the isolated colony smears of samples A-E under 100x oil immersion on the compound light microscope.

3.4 Endospore staining. Microscopic observation of the isolated culture colonies after endospore staining were given in figure 4. It was observed that the bacteria in samples A-E were stained with red colour and the absence of green colour stained endospores were observed. Only vegetative cells were visible. A monolayer of bacteria was observed in samples A-E.

3.5 Catalase test. The catalase test results were observed as shown in figure 5 to determine if the catalase enzyme was present/absent from the isolated colony smears.

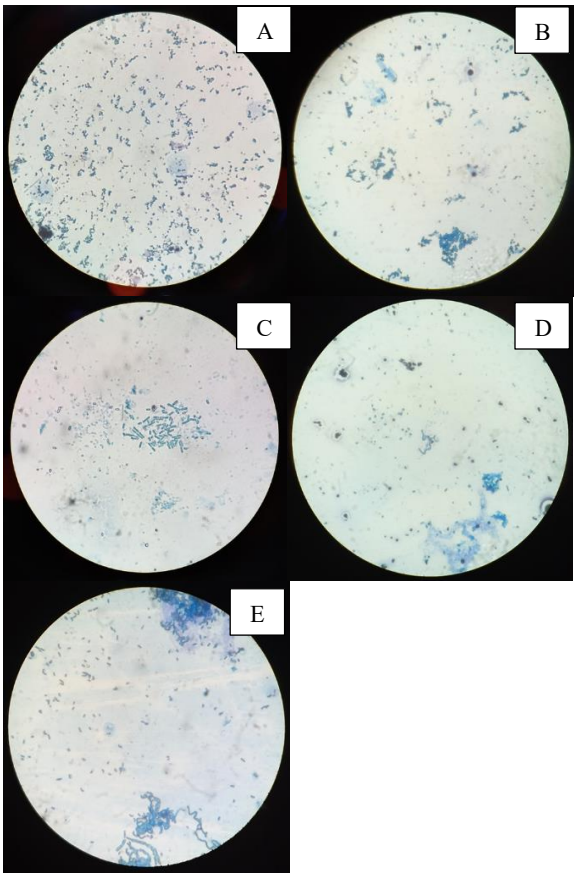
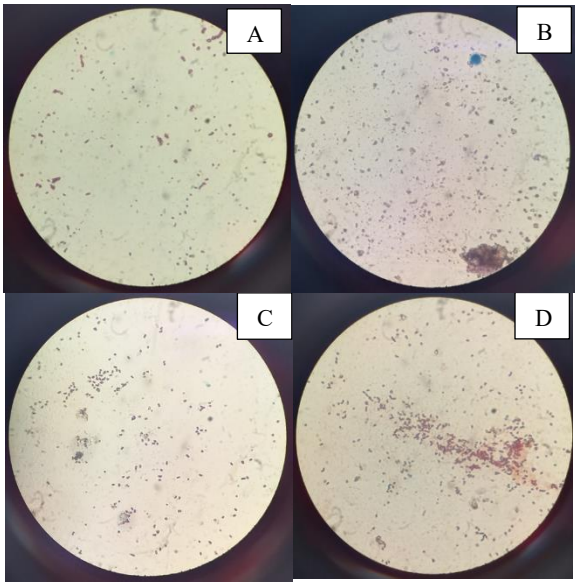


Figure 3. The acid-fast stains of the identified colony smears of samples A-E under 100x oil immersion on the compound light microscope.



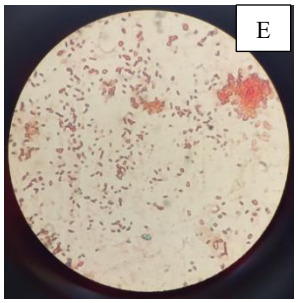


Figure 4. The endospore stains of the isolated colony smears of samples A-E under 100x oil immersion on the compound light microscope.

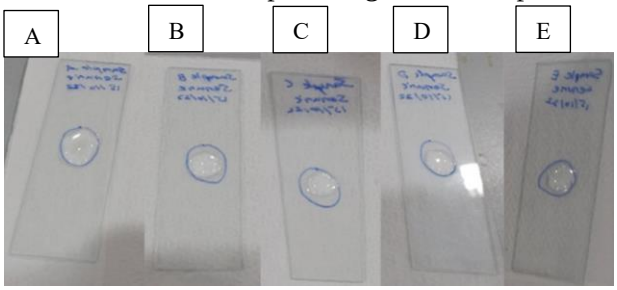


Figure 5. Catalase tests results of each identified colony smears of yogurt samples A-E.

No effervescence was observed from all samples A-E.

3.6 Acid tolerance assay. Acid tolerance of *Lactobacillus* was determined by spectrophotometry analysis by obtaining absorbance values at pH 7.2 and 3 at both 0 and 3 hours. The data was presented in two column charts as given below in figure 6 and 7. A decrease in the mean absorbance readings was noted in samples A-C while an increase in the mean absorbance readings were noted in samples D-E when the readings at 3 hours were compared with that at 0 hours at pH 3.

3.7 Data/statistical analysis. According to table 1 the obtained p-value via SPSS statistical software is 0.945 which is greater than 0.05 (5% level of significance).

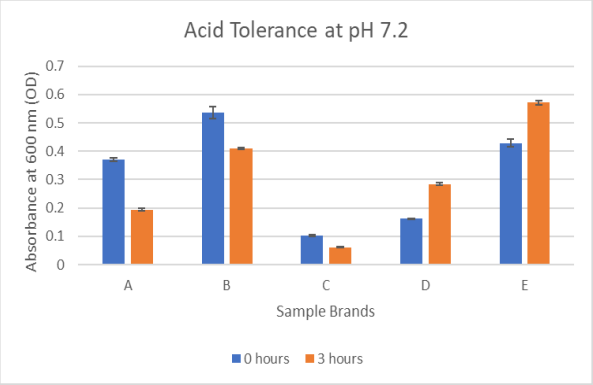


Figure 6. Column chart for the acid tolerance of *Lactobacillus* with the mean absorbance readings for samples A-E at 600 nm at pH 7.2 at 0 hours and 3 hours with the standard deviation.

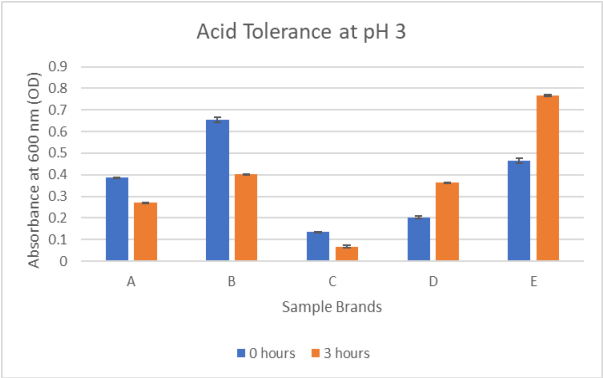


Figure 7. Column chart for the acid tolerance of *Lactobacillus* with the mean absorbance readings for samples A-E at 600 nm at pH 3 at 0 hours and 3 hours with the standard deviation.

Table 1. Statistical analysis by One-Way ANOVA for the mean absorbance readings for samples A-E at 600 nm at pH 3 at 0 and 3 hours.

ANOVA

Absorbance

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.000	1	.000	.005	.945
Within Groups	1.296	28	.046		
Total	1.296	29			

4. Discussion

This research was conducted to determine the acid tolerance of *Lactobacillus*, a bacterium commonly found in dairy products. Bacteria were isolated from five commercially available yogurt samples to determine if the yogurt contains bacteria which can survive under the acidity of the human stomach, an aseptic technique called culturing was used to isolate bacteria from yogurt samples., Morphological analysis of the colonies with a series of biochemical tests were done to confirm the presence of *Lactobacillus*. The acid tolerance assay was performed through spectrophotometry.

The yogurt samples were quadrant streaked on MRS agar and incubated at 37 °C for 48 hours to isolate *Lactobacillus* colonies for further experiments. MRS agar was used as it is a selective agar for the growth of *Lactobacillus* which inhibits the growth of unnecessary bacteria.[26,27] Morphological characteristics of the colonies were observed to determine if the bacteria isolated was *Lactobacillus*. Figure 1.0 presents the colonies formed on sample cultures A-E as creamy/milky in colour, opaque and smooth with the form as circular, elevation as convex and margin as entire. The results were similar to the observations made on the colony morphology of *Lactobacillus* by Afrin *et al* and Rabiei *et al*.[28,29] Based on the evidence it was assumed that the bacteria present in the colonies were indeed *Lactobacillus* spp. For further confirmations, Gram's staining, acid-fast staining, endospore staining and catalase testing were performed. A small fungal growth contamination was observed in sample D-E cultures. It could arise due to unskilled handling, contaminated reagents, media or equipment/glassware, from the hands/skin of the researcher, contaminated air with microorganisms found in incubators.[30] Usage of alternative antimicrobial agents such as nystatin and cycloheximide instead of amphotericin B such as in the studies conducted by Leska *et al* [31] and Nachi *et al* [32] could aid the inhibition of fungi and help prevent culture contamination.

The identified colony smears were subjected to Gram's staining as the first biochemical test to distinguish *Lactobacillus*. Purple-coloured Bacilli (rod-shaped) were seen

under the microscopic examination of the Gram's stain of samples A-E in figure 2 confirming the presence of Gram-positive bacteria. The trapped CV-I complexes inside the cell due to the shrinking of the thick peptidoglycan cell wall pores in the decolorizing step gives rise to the purple colour. Since similar results were obtained in the study conducted by Jose *et al*[33] it confirmed that the observed bacteria were *Lactobacillus*.

The identified colony smears were subjected to acid-fast staining as the second biochemical test. Figure 3 demonstrates blue-colour stained Bacilli in samples A-E thus confirming the presence of non-acid-fast bacteria. It is stated in Siegrist[34] that lactobacilli are acid-fast negative which is supportive of the observations made, thereby it confirmed that the observed bacteria were *Lactobacillus* spp.

The identified colony smears were subjected to endospore staining as the third biochemical test. It was observed that the bacteria in samples A-E in figure 4 were only stained in red and there were no visible green stained endospores. This results revealed that the cells were vegetative cells as it was consistent with the findings made by Malathi *et al*[35] and Goyal *et al* [36] who observed the same for *Lactobacillus* spp. in their research study which further supported the evidence for the presence of *Lactobacillus* in yogurt.

The identified colony smears were subjected to the catalase test as the fourth biochemical test to distinguish *Lactobacillus*. If bacteria contained the catalase enzyme, it would break down hydrogen peroxide into water and oxygen which can be visualized with the production of oxygen bubbles.[37] As shown in figure 5, the catalase test was negative with the absence of bubble formation indicating the presence of catalase-negative bacteria. The research findings of Amin *et al*[37] and Adikari *et al*[38] supports these results as they identified and concluded *Lactobacillus* spp. as catalase-negative with the absence of effervescence in the catalase test. Therefore, it can finally be confirmed that samples A-E contained *Lactobacillus* as all four biochemical tests indicated its presence.

As probiotics are incorporated into a variety of food and enters our body through the GIT, they must possess the ability to tolerate and survive the acids encountered along the way. A pH of 1 was recorded in the stomach when a person is fasting while a pH of around 4.5 is recorded after the consumption of a meal. A pH of approximately 3 was chosen as the test value, to mimic the environmental condition that the bacteria could potentially face the stomach to determine if *Lactobacillus* found in yogurt could withstand it. An incubation period of 3 hours was chosen to mimic the time the bacteria could be exposed to acids in the GIT as the ingestion of food approximately takes up to 3 hours. The assay was also conducted at pH 7.2 as a control and for comparison[39]. From figure 7, it was observed that three of the samples (A-C) had somewhat of a decrease in the quantity of bacteria present at three hours compared with that at zero hours at pH 3 as the mean absorbance had decreased slightly. However, D-E samples showed an increase in the bacterial density at the same pH indicating the growth of bacteria as the mean absorbance had increased. Absorbance was directly proportional to the quantity of bacteria present in a sample. The increase in mean absorbance in samples D-E could account for the survival of *Lactobacillus* at pH 3 similar to the study performed by Faye *et al*[40] due to the usage of either acid tolerant mechanism which pumps out H^+ ions from the cytoplasm via F1-F0-ATPase, utilization of excessive H^+ ions in the GAD system or through the neutralization of H^+ ions via ammonia generation in the ADI system thus allowing the bacterium to withstand acids to grow and multiply. The *Lactobacillus* species likely to be present in yogurt samples were *Lactobacillus casei* strains or *Lactobacillus bulgaricus* strains.[41,42] Studies were conducted on *Lactobacillus acidophilus* by Both *et al* [43] for acid tolerance presented a similar decline in the number of bacterial cells possibly due to the bacterial destruction or the growth being halted in others due to an inhibitory effect on metabolism

and reduction in its viability as a consequence of the disruption of vital elements such as DNA and proteins. due to an intolerable acidic pH.[41] Hence bacteria were unable to multiply and increase in number. However, the p-value obtained from the One-Way ANOVA table was 0.945, which is greater than 0.05 therefore it was considered that there was no significant difference between the mean absorbance readings between zero and three hours at pH 3 and the survival of *Lactobacillus* which was similar to the results obtained by Hassanzadazar *et al*[41] in their study. The decline in bacterial density in the three samples were not significant enough to conclude that they were destroyed due to the intolerability to acids but might have occurred due to a randomized human error. It can be stated that the bacteria were acid tolerant to a certain extent since the results presented a decent capacity for the bacteria to withstand acids but can only be fully confirmed by carrying out further acid tolerant assays which will aid the understanding if the strains are significantly capable of withstanding the stomach's acidity to grow and multiply and provide the health benefits or if the manufacturers need to modify the species to yield a better acid tolerance.

Conclusion

The four biochemical tests with the morphological analysis aided the identification of *Lactobacillus* in yogurt by proving that the bacteria were Gram-positive, non-acid-fast, vegetative cells and catalase-negative. It was determined that the bacteria were capable of withstanding acidity to an extent in the acid tolerant assay and further investigational analysis were necessary to comment on its acid tolerance. The research findings were of significant value as it presented the strains utilized by yogurt manufacturers as potential acid tolerant *Lactobacilli* which benefits consumers.

Acknowledgement

The authors thank BMS (Business Management School) for providing the opportunity to carry out this valuable research at the microbiology laboratory.

References

1 G.G. Syngai, R. Gopi, R. Bharali, S. Dey, G.M.A. Lakshmanan and G. Ahmed. *Journal of Food Science and Technology*, 2016;**53**;921-933.
2 M. Kechagia, D. Basoulis, S. Konstantopoulou, D, Dimitriadi, K. Gyftopoulou, N. Skarmoutsou and E.M. Fakiri. *ISRN Nutrition*, 2013;**2013**.
3 R. Nagpal, A. Kumar, M. Kumar, P.V. Behare, S. Jain and H. Yadav. *FEMS Microbiology Letters*, 2012;**334**;1-15.
4 L.H. Shi, K. Balakrishnan, K. Thiagarajah, N.I.M. Ismail and O.S. Yin. *Tropical Life Sciences Research*, 2016;**27**;73–90.
5 C. Huang, S. Li, L. Huang and K. Watanabe. *Frontiers in Microbiology*, 2018;**9**,1974.
6 G.W. Tannock. *Applied and Environmental Microbiology*, 2004;**70**;3189–3194.
7 K. Śliżewska and A. Chlebicz-Wójcik A. *Biology*, 2020;**9**;423.
8 Y. Widyastuti, A. Febrisiantosa and F. Tidona. *Frontiers in Microbiology*, 2021;**12**.
9 J. Kliks, A. Leśniak, M. Spruch, S. Szołdra and S. Czabaj. *Integrative Food, Nutrition and Metabolism*, **6**(3).
10 S. Misra, D. Mohanty and S. Mohapatra. *Probiotics*, 2022;257-275.
11 N.I.A. Thaidi, L. Rios-Solis and M. Halim. *Probiotics and Prebiotics in Foods*, 2021;135-151.
12 U. Banerjee, T. Halder, R. Malida, R. Panda, T. Halder and G. Roymahapatra, *International Journal of Innovative Practice and Applied Research*, 2017;**7**;57.
13 P. Behare, V.K. Lule and P. Patil. *Food Science*, 2016; 612-616.
14 R. Azat, Y. Liu, W. Li, A. Kayir, D. Lin and W. Zhou, X. Zheng, *Journal of Zhejiang University Science B*, 2016;**17**;597-609.
15 S. Fijan. *International Journal of Environmental Research and Public Health*, 2014;**11**;4745–4767.
16 Y. Guo, X. Tian, R. Huang, X. Tao, N.P. Shah, H. Wei and C. Wan. *Annals of Microbiology*, 2017;**67**; 669–677.
17 N. Guan and L. Liu. *Applied Microbiology and Biotechnology*, 2020;**104**;1-15.
18 C. Wang, Y. Cui and X. Qu. *Archives of Microbiology*, 2017;**200**;1-7.
19 R.K. Duary, V.K. Batish and S. Grover. *Research in Microbiology*, 2010;**161**;404.
20 B.M. Corcoran, C. Stanton, G.F. Fitzgerald and R.P. Ross. *Applied and Environmental Microbiology*, 2005;**71**;3060–3067.
21 G.L. Lorca and F. de Valdez. *Biotechnology Letters*, 2001;**23**;778-779.
22 N. Guan, L. Liu, H. Shin, R.R. Chen, J. Zhang, J. Li, G. Du, Z. Shi and J. Chen J. *Journal of Biotechnology*, 2013;**167**;61-62.

23 R. Wang, N. Li, K. Zheng and J. Hao. *FEMS Microbiology Letters*, 2018;**365**.

24 C. Lyu, W. Zhao, C. Peng, S. Hu, H. Fang, Y. Hua, S. Yao, J. Huang and L. Mei. *Microbial Cell Factories*, 2018;**17**.

25 R.P.K Sahadeva, S.F. Leong, K.H. Chua, C.H. Tan, H.Y. Chan, E.V. Tong, S.Y.W. Wong and H.K. Chan. *International Food Research Journal*, 2011;**18**;1516-1517.

26 M. Soni, H.R. Shah and S.M. Patel. *Asian Journal of Dairy and Food Research*, 2021;**40**;267-272.

27 A. Chowdhury, N. Hossain, N.J. Mostazir, Fakruddin, M. Billah and M.M. Ahmed. *Journal of Bacteriology and Parasitology*, 2012;**3**;2.

28 S. Afrin, S. Akter, S. Begum and M.N. Hossain. *Frontiers in Nutrition*, 2021;**8**.

29 M. Rabiei, G. Zarrini and M. Mahdavi. *Advanced Pharmaceutical Bulletin*, 2020;**10**;125–129.

30 J. Ryan. *Understanding and Managing Cell Culture Contamination*. *Technology Networks*, 2021.

31 A. Leska, A. Nowak and I. Motyl. *Agriculture*, 2022;**12**;1562.

32 I. Nachi, I. Fhoula, I. Smida, H. Ouzari and M. Hassouna. *Annals of Microbiology*, 2018;**69**;29–40.

33 N.M. Jose, C.R. Bunt and M.A. Hussain MA. *Microorganisms*, 2015;**3**;198-212.

34 J. Siegrist. *Lactobacilli Basics, Testing & Identification*, no date.

35 U. Malathi, G. Praveenkumar and S. Vuppu. *International Journal of Drug Development and Research*, 2015;**7**;94-100.

36 R. Goyal, H. Dhingra, P. Bajpai and N. Joshi. *African Journal of Biotechnology*, 2012;**11**;14448-14452.

37 U.S. Amin, A.K.M. Kober, N. Akter, M.A. Hossain and E.A. Rana. *Journal of Modern Agriculture and Biotechnology*, 2022;**1**;2.

38 A.M.M.U. Adikari, H. Priyashantha, J.N.K Disanayaka, D.V. Jayatileka, S.P. Kodithuwakku,, J.A.M.S. Jayatilake and J.K. Vidanarachchi. *Heliyon*, 2021;**7**.

39 A.H.S. Soliman, A.M. Sharoba, H.E.M. Bahlol, A.S. Soliman and O.M.M. Radi. *Middle East Journal of Applied Sciences*, 2015;**5**;96.

40 T. Faye, A. Tamburello, G.E. Vegarud and S. Skeie. *Journal of Dairy Science*, 2012;**95**;558-566.

41 H. Hassanzadazar, A. Ehsani, K. Mardani and J. Hesari. *Veterinary Research Forum*, 2012;**3**;181–185.

42 S.E. Evivie, A. Abdelazez, B. Li, S. Lu, F. Liu and G. Huo. *Frontiers in Microbiology*, 2020;**11**.

43 E. Both, E. Gyorgy, C.Z. Kibedi-Szabo, E. Tamas, B. Abraham, I. Miklossy and Lanyi S. *UPB Scientific Bulletin, Series B*, 2010;**72**;40.